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CIRCULATING AUTOANTIBODIES IN HUMAN TRAUMATIC SPINAL CORD INJURY SUBJECTS AND THEIR RELATIONSHIP TO THE DEVELOPMENT OF NEUROPATHIC PAIN

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by

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A

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Dedication

This is dedicated to the people with spinal cord injury who have generously participated in research.

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Abstract

CIRCULATING AUTOANTIBODIES IN HUMAN TRAUMATIC SPINAL CORD INJURY SUBJECTS AND THEIR RELATIONSHIP TO THE DEVELOPMENT OF NEUROPATHIC PAIN

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Background:

Approximately 17,500 spinal cord injuries (SCI) occur yearly in the U.S. causing considerable morbidity and mortality. Neuropathic pain (NP) ensues in 40-70% of SCI. An autoimmune response resulting from disruption of the blood-spinal cord-barrier may be a contributor to NP. However, the relationship between autoantibodies and NP after SCI in humans has not been thoroughly characterized nor have autoantigens been identified. Glial fibrillary acidic protein (GFAP) and collapsin response mediator protein2 (CRMP2) were identified as candidate autoantigens. The hypothesis is that proteins from the injured spinal cord released by SCI trigger autoantibody production which can lead to the development of NP.

Results:

The presence of autoantibodies to GFAP (GFAPab) and CRMP2 (CRMP2ab) and their correlation to the development of NP was evaluated. GFAPab was present in 21 of 38 (55%) acute SCI, 34 of 80 (43%) chronic SCI. CRMP2ab was present in 8/35 (23%) acute SCI patient plasma samples. Complement C3 and C5 were elevated in acute SCI. Peak autoantibody levels were detected at 16±7 days post injury. The peak plasma GFAPab levels were higher in patients that subsequently developed NP versus those who did not (T=219, p=0.02). Receiver operator characteristic curve analysis shows that plasma GFAPab levels had an area under the curve of 0.71 (95% CI, 0.53-0.89 p=0.03) for the discrimination of patients

that developed NP within 6 months after injury. Patients with GFAPab and/or CRMP2ab had a 9.5 times increased odds of developing NP.

Discussion:

Results show that SCI triggers an autoimmune response leading to production of autoantibodies. The 16±7 day level of GFAPab post-SCI is a predictor of the development of NP. The levels of GFAPab returned to levels found in healthy volunteers by 96±54 days post-injury. A panel of GFAPab and CRMP2ab showed 9.5 times increased odds of developing NP (95% CI, 2.08-43.50, p=0.006). Future studies will examine the possibility that other autoantibodies contribute to the development of NP. Measuring GFAPab and CRMP2ab post-SCI may help identify patients at risk for subsequently developing NP. A reduction of GFAPab and/or CRMP2ab in the acute stages of injury may decrease the likelihood for developing NP.

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Abbreviations

AD	Alzheimer's disease
ASIA	American Spinal Cord Injury Association
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
BDP	Breakdown products
bFGF	Basic fibroblast growth factor
BSCB	Blood-spinal cord barrier
C3	Complement component C3
C5	Complement component C5
CALR	Calreticulin
CD	Cluster of differentiation (e.g., CD4+ or CD8+ cells)
Cdk5	Cyclin-dependent kinase 5
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
CRMP2	Collapsin response mediator protein 2
CRMP2ab	CRMP2 autoantibody (determined by immunoreactivity after validation)
CSPG	Chondroitin sulfate proteoglycans
DAMPs	Damage associated molecular patterns
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding; two arms of the antibody molecule that contain antigen-binding activity

Fc	Fragment crystallizable; constant region of the antibody that does not bind antigens, interacts with effector molecules and cells.
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFAPab	GFAP autoantibody (determined by immunoreactivity after validation)
GM1 ganglioside	Monosialotetrahexosylganglioside (prototype ganglioside)
GSK3β	Glycogen synthase kinase-3β
HIV-1	Human immunodeficiency virus-1
HRP	Horseradish peroxidase
IASP	The International Association for he Study of Pain
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IL	Interleukin
IPG	Immobilized pH gradient
IVIG	Intravenous immunoglobulin
ISNCSCI	International Standards for Neurological Classification of Spinal Cord Injury
LC-MS/MS	Liquid chromatography-mass spectrometry
MAC	Membrane attack complex
MBL	Mannose binding lectin
MBP	Myelin basic protein
microBCA	micro bicinchoninic acid
mRNA	Messenger RNA (ribonucleic acid)
Myc-DDK	Myc tag from the c-myc gene and DDK polypeptide protein tag
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMO	Neuromyelitis Optica
NP	Neuropathic pain

P38 MAPK	p38 mitogen-activated protein kinase
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBST	Phosphate buffered saline with Tween-20
pH	Potential of hydrogen
pI	Isoelectric point
PMSF	Phenylmethane sulfonyl fluoride
PMT	Photomultiplier tube (setting voltage on the Typhoon Trio)
PNS	Peripheral nervous system
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
ROC	Receiver operator characteristic curve
ROS	Reactive oxygen species
S/N	Signal to noise ratio
S-LANSS	Leeds Assessment of Neuropathic Symptoms and Signs
SCI	Spinal cord injury
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sema3A	Semaphorin 3A
SLE	Systemic lupus erythematosus
T1	Time 1 (less than 2 days after spinal cord injury)
T2	Time 2 (8-30 days after spinal cord injury)
TBM	Tetramethylbenzidine
TBST	Tris-buffered saline solution containing 1% Tween-20
Tc	Cytotoxic T cells
TGF	Transforming growth factor
Th	Helper T cells

TNFα	Tumor necrosis factor alpha
TRPV1	Transient receptor potential vanilloid 1 channels

1. Rationale, Hypothesis, Specific Aims and Significance

Rationale: Chronic neuropathic pain is a debilitating condition that commonly occurs after spinal cord injury (SCI). More than half of those with SCI-related neuropathic pain describe neuropathic pain as their primary problem, more significant than their loss of motor function.[1] Forty to seventy percent of those with SCI develop neuropathic pain, yet currently available treatments (e.g., gabapentin and pregabalin) are only partially effective, and only in some patients.[2] Additionally, there are no predictors of post-SCI neuropathic pain.[3] A potential contributor to SCI-induced neuropathic pain and inhibitor of recovery is the autoimmune response to damaged central nervous system (CNS) tissue.

The conditions for an autoimmune response occur as the primary trauma to the spinal cord disrupts the blood-spinal cord barrier (BSCB). This disruption allows previously sequestered CNS molecules to enter the systemic circulation where they may elicit an autoimmune response. Increased permeability of the BSCB also allows circulating immune cells to infiltrate and access the injury site. The increased access of circulating immune cells to the injury site initiates the potential for an autoimmune response to ensue.[4] Rodent models using both rats and mice have demonstrated that SCI can lead to stimulated T- and B-lymphocyte responses leading to the production of immunoglobulin G (IgG) autoantibodies to CNS proteins.[5, 6] These IgG autoantibodies from injured subjects can exacerbate tissue injury by causing ongoing inflammation, hinder recovery and promote chronic pain. Consistent with this, a study utilizing a constriction model of SCI showed less mechanical allodynia and hyperalgesia in rats lacking functional T cells compared to controls.[7] The recognition of antigen by T cells and major histocompatibility complex II generates inflammatory mediators and facilitates the presence of chronic pain.[8] Furthermore, SCI in B cell knockout mice that are incapable of generating an antibody response were shown to have better locomotor function and smaller lesion volume

compared to SCI wild-type mice.[9] Additionally, injecting purified IgG obtained from SCI mice into uninjured mice resulted in spinal cord pathology and decreased motor function, similar to that of the SCI mice.[5] These studies demonstrate negative effects of autoimmune responses in rodent models. Relatively few human studies have identified autoantibodies produced after SCI and, for the most part, these studies have been performed using directed searches (primarily against myelin proteins, gangliosides and glycoproteins) limiting their diagnostic and prognostic potential for neuropathic pain.[10-19] While neuroinflammation is recognized as an ongoing process after SCI, autoantibody discovery after human SCI has not been thoroughly explored.[20, 21] Ongoing inflammation due to an autoimmune reaction to CNS antigens may contribute to neuropathic pain.

Hypothesis: proteins from the injured spinal cord released by SCI trigger autoantibody production that can lead to the development of neuropathic pain. Two specific aims are outlined to test the above hypothesis.

Specific Aim 1**:** To test whether SCI elicits an autoantibody response and identify the antigen(s) that triggers this response in human SCI subjects**.**

Specific Aim 2: To statistically compare whether the presence of autoantibodies to specific antigen(s) correlates with the development of neuropathic pain in human SCI subjects.

Inflammatory processes such as astrogliosis, abnormal cytokine production and activation of complement are known to occur after SCI and have been associated with neuropathic pain.[22-26] Mouse models have identified autoantibodies produced after SCI to CNS antigens.[27] However, the presence of autoantibodies to CNS proteins in human SCI has not been well documented, nor have the antigens predictive of neuropathic pain been established. Western blot-based approaches were utilized to explore the presence of autoantibodies in human plasma following SCI. Investigations were performed to test for an association between the presence of the autoantibodies and neuropathic pain. Using this

unbiased screening, newly enhanced immunoreactivity to collapsin response mediator protein 2 (CRMP2) and glial fibrillary acidic protein (GFAP), both CNS antigens, was detected in patients' plasma after SCI. Additionally, complement components C3 and C5 were increased in patients' plasma after SCI. These data suggest that release of CRMP2 and GFAP after SCI may trigger an autoimmune response that contributes to neuropathic pain.

Further studies were conducted to verify the antigen specificity. The time course of circulating GFAP autoantibodies (GFAPab), CRMP2 autoantibodies (CRMP2ab) and complement C3 and C5 levels in SCI patients and whether the presence of GFAPab, CRMP2ab, C3 or C5 correlated with subsequent development of neuropathic pain was evaluated. Plasma complement C3 levels at 6.4 ± 1 days and GFAPab at 16 ± 7 days were correlated; C3 and CRMP2ab levels at 16 ± 7 days were correlated. Analysis identified an association between the presence of GFAPab at 16 ± 7days post-SCI and the development of neuropathic pain within 6 months post-SCI. Combining the GFAPab plus CRMP2ab into a panel determined that the presence of these autoantibodies increased the odds of developing neuropathic pain 9.5 times compared to those without GFAPab and CRMP2ab (95% CI, 2.08-43.50, p=0.006). When controlling for age, gender, body mass index, complete injury, and cervical level, the presence of GFAPab and/or CRMP2ab remained a significant factor in predicting neuropathic pain within 6 months of SCI (OR 15.3, 95% CI 1.9 to 125, p=0.01).

Significance: These data suggest the autoimmune response to GFAP and CRMP2 is a contributor to chronic pain following SCI in humans. If these results are confirmed, GFAPab and CRMP2ab may be used as a predictor of neuropathic pain. Treatments aimed at the removal of GFAPab early post-injury may be able to prevent the development of neuropathic pain. This suggests screening for autoantibody production may initiate new strategies for preventive therapies.

Chapter 1. Introduction

1.1. Description and course of spinal cord injury

Approximately 17,500 SCI occur annually in the U.S.[28] The World Health Organization estimates that the worldwide incidence of SCI is up to 500,000/year. Compared to those without injury, people with SCI are 2 to 5 times more likely to experience premature death.[29] Approximately 80% of injuries occur in males. The average age at time of SCI is 42 years; 59% of injuries result in tetraplegia (45.8% incomplete tetraplegia, 13.2% complete tetraplegia), 40.6% paraplegia (20.9% incomplete paraplegia,19.7% complete paraplegia) and 0.4% are neurologically normal by hospital discharge.[28] The leading causes of SCI are motor vehicle accidents, falls, assault and sports-related injuries.

Spinal cord injury can be segregated into two stages, 1) the primary injury from the initial mechanical insult causing damage to the spinal cord resulting in cell death and the activation of the inflammatory response; and, 2) the secondary injury caused by the vascular and immune responses associated with a persistently permeable BSCB. Loss of function occurs as a result of loss of conduction of sensory and motor signals across the lesion site. Damage to axons of spinal sensory neurons or dorsal nerve roots causes interference of ability of afferent signals to reach the brain, and damage to axons of motor neurons or ventral nerve roots inhibits efferent signals from reaching muscles.

1.2. Level and classification of injury (complete or incomplete)

SCI level of injury is classified according to the most caudal spinal cord segment (cervical, thoracic, lumbar, or sacral) that is functionally normal. Injury to the cervical spinal cord results in tetraplegia. Tetraplegia manifests as malfunction of arms, legs, thoracic and pelvic region. Paraplegia refers to loss of function of thoracic, lumbar or sacral spinal cord

segments as a result of spinal cord damage. The specific spinal segment injured dictates the level of function. Paraplegia includes preserved upper extremity function.

SCI degree of neurological impairment is classified as complete or incomplete. A complete SCI is one in which no motor or sensory function exists below the level of the lesion. An incomplete injury is one in which some motor or sensory function exists below the lesion. The most accepted tool used to classify subjects' injury is the *International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI)* formerly referred to as the *American Spinal Cord Injury Association (ASIA)* exam which was based on the *Frankel Classification*.[30] The exam captures bilateral upper and lower extremity motor function based on myotome, and sensory function by dermatome location. Muscle grade is from 0 (total paralysis) to 5 (active movement against full resistance). Sensory points are graded as 0 (absent), 1 (impaired) or 2 (normal). In order to be motor incomplete, the SCI subject must have sacral sparing and either voluntary anal sphincter contraction or motor function preserved for at least three levels below the motor level. In addition to classifying the degree of impairment the ISNCSCI assigns motor and sensory levels at the most caudal segment of the spinal cord where the patient is bilaterally normal. Of note, this is not necessarily the same as the level of the lesion as it refers to normal *function*. Generally, patients' receive a summary ISNCSCI score based on the spinal segment and degree of completeness. For instance, a person who has normal motor function and is able to fully extend their wrist against normal resistance (extensor carpi radialis longus and brevis, C6) and has normal sensory function to the thumb and index finger, but no function below that level (complete, A) would be defined as a "C6 A". Not all SCI are neatly classified and a patient's exam may change over the course of the injury, in part related to edema/swelling.

Classification is useful as a gauge to measure patients' improvement or decline in function, to identify sensory aberrations, to anticipate lifestyle modifications, rehabilitation

capability and secondary complications. Patients have varying degrees of recovery related to the neurological level of injury and degree of impairment. For instance, someone with a cervical complete injury has less chance of recovery of function and independence, and more complications than someone with a thoracic incomplete injury.[31] The rate of motor recovery is highest during the first 3 months and plateaus around 6 months post-SCI.[32] Ninety-four percent of patients with complete injuries remained complete 1 year to 5 years post-SCI and, at most, 2% of those with motor incomplete injuries improved neurologically.[32]

Two recent studies have been performed evaluating the ability of early magnetic resonance imaging (MRI) biomarker measurements for predicting short term neurological outcome after cervical and thoracic SCI. Using a data-driven tool, nonlinear principal component analysis to detect statistical patterns, neurological impairment was predicted using MRI biomarker measurements demonstrating that the Brain and Spinal Injury Center (BASIC) score (lesion/no lesion) was predictive for short term outcome (ISNCSCI score at hospital discharge) for both cervical SCI patients when correcting for interactions from surgical decompression and spinal cord compression, and thoracic SCI patients.[33, 34] This type of objective biomarker measurements from imaging or other biological sources are helpful in classifying injury and in evaluating response to existing and experimental treatments.

1.3. Acute and chronic spinal cord injury

Damage to the cord begins at the time of trauma and the pathology is progressive. There is no precise demarcation between the acute and chronic phases of injury.[35] The distinction is often contextual. Generally, the acute hospital stay when a patient is being stabilized is considered acute. By the time a patient has lived with an injury for 6 months the injury is considered in the chronic phase of recovery.[36, 37] During the acute phase of

injury care focuses on stabilization to prevent further injury and medical management to prevent secondary complications. Rehabilitation to maximize remaining function is initiated as the patient recovers. Secondary conditions associated with SCI and their overall health status affect the injured person's ability to participate in rehabilitation.

1.4. Secondary conditions associated with spinal cord injury

People with SCI have considerable morbidity including pain (nociceptive and neuropathic), depression, infections, skin breakdown, deep vein thrombosis, spasticity, contractures, autonomic dysreflexia, bone demineralization, muscle atrophy, bowel, bladder and sexual dysfunction in addition to loss of motor and sensory function.[38, 39] These secondary morbidities contribute to diminished quality of life and reduced life expectancy.[40-42] The scope of this work focuses primarily on neuropathic pain resulting after SCI.

1.5. Innate and adaptive response

The innate immune response is the initial, non-specific immune response which defends the host against pathogens, eliminates dead or damaged cells and initiates tissue repair. This non-specific immune response initiated immediately after SCI causes vasodilation, edema, cytokine and chemokine production and an influx of leukocytes and polymorphonuclear cells as the body works to repair damaged tissue.[43] The adaptive immune response is antigen-specific and therefore requires time to respond. The innate response is efficient in recognizing specific patterns. For instance, the innate system uses Pattern Recognition Receptors (PRR) that recognize motifs found on the surface of bacteria and other pathogens to identify them for destruction. The innate system also recognizes damage-associated molecular patterns (DAMPS) found on cellular debris and

initiates its removal.[44] The innate immune response activates the complement system to enhance antibodies' and phagocytes' ability to clear cellular debris and pathogens.[9, 45] The opsonization (coating) of pathogens by complement facilitates their uptake by phagocytic antigen presenting cells expressing complement receptors. This readies the antigen for T cells. Conventional dendritic cells take up antigen in tissue, process it to generate a peptide antigen that can activate T cells and induce an adaptive immune response.[46, 47] After SCI, these antigens could be fragments of CNS proteins. There are two types of antigen-specific lymphocytes, T cells and B cells. B cells are produced in the bone marrow, have B cell receptors on their surface that are specific for antigens, and produce antibodies.

The adaptive immune response is induced as T cells and B cells receive antigen stimulation; T cells differentiate into T effector cells (helper (Th) and cytotoxic (Tc)) and B cells differentiate into antibody-secreting plasma cells. Activated antigen presenting cells (dendritic cells) present antigen and prime T cells in peripheral lymph nodes or, as in SCI with severe inflammation, naïve T cells can be activated within the CNS. [48] Disruption of gut microbiota is also involved with priming T cells, activation of B cells and autoimmune responses.[49, 50] T cells are activated and stimulated to release cytokines. CD4+ T cells secrete cytokines in the CNS; Th1 secrete IFN-γ and Th17 secrete IL-17, IL-21 and IL-22.[48] CD8+ T cells secrete TNF-α, IFN-γ.[51] The autoimmune disease systemic lupus erythematosus (SLE) is associated with antigen presentation by dendritic cells in association with CD4+ T cells; additionally, CD8+ T cell deficient mice are resistant to the development of SLE showing T cells can contribute to autoantibody production with cytokine production.[51]

Mature B cells are released into the circulation and lymphatic system from the bone marrow as naïve B cells. Upon receiving stimulation from antigen in the presence of Th cells in lymph nodes a germinal center reaction results in clonal expansion, B cell class

switching, somatic mutation and the production of 1) activated memory B cells that are long-lived and 2) plasma cells that secrete antigen-specific antibodies.[48]

1.6. Autoantibody

Antibodies bind specifically to the antigen to generate the immune response and to recruit cells to destroy the antigen once it is bound by the antibody. Autoantibodies are antibodies directed against self-antigens. During typical lymphocyte differentiation, some lymphocytes develop that have affinity for self-antigens. These lymphocytes are removed or have such low affinity to self-antigens that they are of little significance. A state of self-tolerance exists where the immune system does not attack normal self-tissues. Autoimmunity occurs when this system malfunctions.[52] Autoimmune disease results from mounting of an immune response to a self-antigen(s). For instance, multiple sclerosis is the result of auto-reactive T cells against CNS antigens causing the formation of sclerotic plaques and destruction of myelin sheaths resulting in muscle weakness and ataxia.[53, 54] Pathology occurs when there is a sustained reaction and myelin is destroyed.[55] Methods of tolerance include the sequestration of antigens where they are not accessible to the immune system. Trauma that increases the permeability of the BSCB and tissue damage allowing previously sequestered antigens to become accessible abrogates this sequestration. The release of CNS antigens can activate ignorant latent T and B cells. Autoantibodies may be produced by B cells when self-tolerance malfunctions and the body destroys otherwise normal tissue that the immune system perceives as foreign.[56]

Autoantibody binding triggers B and T cell reactions such as the release of inflammatory mediators (e.g., IL-1β, IL-6, TNFα, lymphotoxin) that excite nociceptors, or cause a conformational change or change in expression of receptors or channels, or induce nerve cell damage resulting in sensitization of nociceptors inducing neuropathic

pain.[57-61] However, there is controversy about whether autoantibodies produced after SCI are neuroprotective or neurotoxic. For example, enhancing T-lymphocyte response to antigens at the injury site has been shown to improve neuronal survival in an optic nerve injury model.[62] Additionally, intravitreal injection of glutamate or ferrous ions (free radicals) to nude mice possessing no mature T cells produced significant retinal ganglion cell loss that was not seen in wild type mice demonstrating neuroprotection from T cells.[63] A murine retinal ganglion cell study attributed credit to T cells for protection against thrombin-induced toxicity because of T cells' ability to transcribe thrombin inhibitor anti-thrombin III.[64] It was previously demonstrated that after crush injury, retinas of rats treated with the thrombin inhibitor NAPAP (N-α-(2-naphthylsulphonylglycyl)-4-(D,L)-amidinophenylalanine piperidide acetate salt) had more intact retinal ganglion cells compared to vehicle treated rats.[65]

Efforts to utilize the immune response in order to induce neuroprotection after SCI are under study. Nogo is an axonal growth inhibitor protein. Anti-Nogo-A antibodies infused intrathecally have facilitated axon regeneration in rats and monkeys.[66] It has been proposed that the anti-Nogo-A antibody induces T cell mediated neuroprotection.[67] A safety and feasibility clinical trial of humanized anti-Nogo-A antibody (Novartis Pharmaceuticals) was recently conducted in 52 acute SCI patients; the results are pending.[68]

Alternatively, antibodies created in response to myelin damage at the site of injury are believed to result in secondary damage.[6] Myelin basic protein (MBP) is a major structural protein in myelin. MBP-reactive T cells propagate the inflammatory process by producing cytokines. Chronic SCI patients have been shown to have high levels of MBP-reactive T cells.[69] GM1 ganglioside-specific autoantibodies, as well as cytokines IL-2 and TNFα, which promote axonal dysfunction and demyelination, were elevated in chronic SCI patients.[19]

1.7. Neuropathic Pain after Spinal Cord Injury

Pain serves a protective function in that it signals the body to prevent or notifies the body of tissue injury. Virtually all patients experience pain due to trauma after an SCI. However, neuropathic pain is a distinct type of pathological pain that persists after wound healing. Prevalence rates of neuropathic pain after SCI range from 26% to 96% of patients.[3] Using more consistent definitions of neuropathic pain the estimated range is narrowed to 40% - 70% of SCI patients. [2, 70, 71] Once neuropathic pain occurs it tends to become chronic.

Neuropathic pain is defined as “pain caused by a lesion or disease of the somatosensory system”.[70, 72, 73] The current definition was derived from the International Association for the Study of Pain (IASP) that defined neuropathic pain as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system”.[74] The IASP proposed a uniform classification system to define pain: nociceptive (musculoskeletal and visceral) and neuropathic (at level and below level of injury). The International Spinal Cord Injury Pain Classification system offers a tiered system of classification of pain types.[70] In addition to necessitating a CNS lesion, these neuropathic pain definitions include characteristics requiring that 1) sensory deficits exist within the pain distribution; 2) allodynia and/or hyperalgesia exist within the pain distribution and 3) the patient verifies that neuropathic pain descriptors (e.g., hot-burning, tingling, pins and needles, etc.) depict their pain. In contrast, musculoskeletal pain is characterized as a dull aching pain that gets worse with movement, and is often related to overuse injuries (i.e. transfers relying on only arm and shoulder muscles).

Pain limits physical function and ability to rehabilitate, infringes on work and social activities, and reduces quality of life. Approximately 75% of SCI patients with neuropathic pain described it as a sharp, shooting continuous pain that is stimulus-independent; it is

associated with allodynia (normally non-painful stimuli evoking pain), hyperalgesia (increased sensitivity to noxious stimuli), and paresthesia (pins and needles sensation).[2] The factors that are associated with, or causal of, the development of chronic neuropathic pain in SCI have not been fully determined.

Neuropathic pain and spasticity (exaggerated muscle tone with increased tendon reflexes and clonus) are both aftereffects of maladaptive neuronal plasticity resulting from injury, but musculoskeletal pain due to spasticity differs from neuropathic pain and is differentiated through careful assessment.[75] Multiple factors including psychological, pain location, pain descriptors and onset, injury characteristics, physical condition and environment must be taken into account when evaluating pain.[75] Distinguishing the type of pain experienced after SCI (neuropathic, nociceptive, or other) is important in determining the appropriate treatment. Unfortunately, neuropathic pain is frequently refractory to existing treatments.[76-78] Gabapentin and pregabalin, commonly used medications for SCI neuropathic pain, provide partial relief.[79, 80] In addition to unsatisfactory pain relief, existing treatments have undesirable side effects.[81] For example, these medications are known to cause drowsiness, weakness, fatigue, cognitive issues, constipation, dry mouth and headache. It is difficult to predict which SCI patient is at risk for developing neuropathic pain. Notwithstanding the above definition of neuropathic pain there is no universally accepted objective test for diagnosing neuropathic pain, and a clinical diagnosis of neuropathic pain remains the standard on which most tools are based.[70, 82-84] There are ongoing international efforts for standardizing the definition and grading of neuropathic pain.[70, 77, 83, 84]

1.8. Pain Pathways

Pain is sensed by specialized sensory receptors (nociceptors) that transmit signals from noxious stimuli along A delta (Aδ) and C fibers to the dorsal horn of the spinal cord.

Thermoreception is also transmitted through Aδ and C fibers. There are three types of primary afferent nerve fibers, Aδ, C and Aα/β fibers. Aα/β fibers are large diameter myelinated fibers that have a low threshold for activation and conduct signal rapidly and transmit non-noxious stimuli/mechanoreception.[85] Aδ fibers transmit sharp, fast pain. They are small diameter myelinated fibers. C fibers are unmyelinated and small and therefore slow conducting. They carry dull, long lasting pain and are responsible for perceiving burning pain. Most primary afferent fibers enter the spinal cord through dorsal roots at their level of origin or 1-2 segments above or below, although some unmyelinated afferent fibers enter the spinal cord though ventral roots.[85] Generally, C fibers terminate in laminae 1-2 and Aδ fibers in laminae 1, 2, 5 and 6. [85] The major pathways that transmit pain signals are the spinothalamic tract and the spinoreticular tract. Secondary afferent fibers decussate close to the level of entry and ascend on the contralateral spinothalamic tract to the thalamus to third order neurons which end in the somatosensory cortex. The spinoreticular tract is implicated in the emotional component of pain; it also ascends on the contralateral side and after reaching the reticular formation of the brainstem goes to the thalamus and hypothalamus and then to the cortex. There are descending pathways that inhibit pain transmission, these are central to Melzack and Wall's gate control theory of pain sensation, and the ascending/descending pain transmission system.[86, 87] The gate control theory proposes that non-noxious sensory input from Aα/β fibers activates inhibitory neurons which inhibit pain transmission (input from the C or Aδ fibers). The gate is opened or closed depending on the balance of the large (Aα/β) and small (C or Aδ) fiber input; prolonged high-intensity stimulation disturbs the balance which results in removal of presynaptic inhibition of sensory inputs and opens the gate. The gate control theory proposes that peripheral sensory and central inputs cause brain activity to reach a threshold evoking pain.[87] The ascending/descending pain transmission system is made up of areas that have high concentrations of opioid receptors

and endogenous opioids; descending pathways project to the dorsal horn using noradrenaline and serotonin to inhibit pain. The system includes the upper brain stem periaqueductal gray matter, the locus coeruleus the nucleus raphe magnus and nucleus reticularis gigantocellularis.[88] Pain perception and processing occurs in multiple areas of the brain including the thalamus, the somatosensory, insular, anterior cingulate, and prefrontal cortex. Multiple mechanisms are involved in the development and maintenance of neuropathic pain.

1.9. Mechanisms involved in neuropathic pain

Injury to the spinal cord results in damage and disruption of the pathways that transmit signals from peripheral sensory receptors to the dorsal horn of the spinal cord and then ascend to the brain. Studies in animals have identified multiple mechanisms contributing to the development or maintenance of neuropathic pain, however these mechanisms are not fully understood.[89] Neuropathic pain mechanisms can be interrelated. These include increased spontaneous activity of pain nociceptive neurons, sprouting of C and Aδ fibers, altered expression of ion channels on neurons in the pain pathway, removal of inhibitory inputs, altered release and reuptake of the excitatory neurotransmitter glutamate, altered expression of excitatory neurotransmitter receptors, increased efficacy of “silent synapses” or newly formed synaptic circuits and synaptic reorganization, and increased firing of dorsal root ganglion neurons.[90-101] These mechanisms do not occur in isolation, and central components of developing neuropathic pain include neuronal hyperexcitability and inflammation.[102, 103]

Peripheral sensitization (enhanced response to stimuli by the nociceptor) and central sensitization (enhanced response to painful stimuli in the spinal cord dorsal cord neurons) can occur after SCI.[104] Previously silent nociceptive neurons become responsive after a prolonged period of stimulation, and nociceptive neurons are exposed to

excitatory neurotransmitters including substance P and calcitonin gene-related peptide creating peripheral sensitization and pain.[105-109] Peripheral sensitization causes increased frequency of nerve impulses leading to hyperexcitability from prolonged release of glutamate in the dorsal horn and subsequent central sensitization and pain.[104]

Neuronal hyperactivity, defined as enhanced spontaneous neuronal excitability or enhanced neuronal responses to sensory stimuli resulting in pain hypersensitivity, is essential for the development and maintenance of neuropathic pain. [110] In a study of 15 people with complete thoracic SCI at-level hypersensitivity to cold, touch and pinprick was associated with neuropathic pain.[111] After SCI multiple alterations can occur causing neuronal hyperexcitability in the dorsal horn resulting in neuropathic pain.[104] Using a sensorimotor test to evaluate mechanical allodynia in rodents, correlations have been found between neuronal hyperactivity and neuropathic pain as demonstrated by 1) decreased threshold for paw withdrawal with simultaneous increased firing of the dorsal horn sensory neurons and 2) increase in ion channel expression with neuropathic pain behaviors.[110]

Inflammation in the spinal cord which leads to secondary tissue damage is an important contributor to neuropathic pain.[112, 113] CNS glial cells (microglia, astrocytes and oligodendrocytes) are involved in the initiation and continuation of neuropathic pain. For instance, blocking glial activation in rats prevented neuronal and glial activation of a phosphorylated MAPK (p38 MAPK) and reduced neuronal hyperexcitability and mechanical allodynia.[104, 114] Glial cell activation has also been identified in human spinal cord tissue. A study of post-mortem human spinal cords including 11 SCI and 2 traumatic brain injury (TBI) control spinal cords evaluated spinal cord tissue obtained from people who died 30 minutes to 5 days post-SCI. Activated microglia, IL-1β, IL-6 and TNFα, all associated with inflammation, were found near the lesion and in the spinal cord as early as 30 minutes post-SCI, but no expression was found in the spinal cords of people

who died secondary to TBI without SCI.[115] Serum levels of IL-6, and TNFα were elevated in people with SCI and these cytokines were further elevated in people with pain or who had active infection.[16] The presence of inflammatory mediators and the fact that blocking them alleviates pain provides support for inflammation as a mechanism for neuropathic pain.

Activated astrocytes and microglia release pro-inflammatory cytokines, neurotransmitters, and reactive oxygen species (ROS). This results in increased blood-spinal cord barrier (BSCB) permeability, and abnormal function of ion channels and receptors. Activation of voltage-gated calcium channels and NMDA receptors result in an increase in intracellular calcium ions, this leads to activation of multiple downstream kinase pathways (e.g., calcium-calmodulin-dependent kinase II, protein kinase C, protein kinase A) which perpetuate neuronal hyperexcitability of dorsal horn sensory neurons.[110]

Glial cells, including microglia and astrocytes are involved in hyperexcitability and neuropathic pain. Glial cells maintain gamma-aminobutyric acid (GABA) and glutamate concentrations in the spinal cord contributing to both release and uptake in order to maintain extracellular concentrations.[89] GABA is a neurotransmitter that acts on inhibitory interneurons; loss of GABA inhibition results in hyperexcitability in dorsal horn neurons and neuropathic pain.[89, 116] Glutamate, an excitatory neurotransmitter, contributes to sensitization. After painful stimuli, intracellular calcium concentration increases in astrocytes which in turn cause an increase in calcium-dependent glutamate. High intracellular calcium concentrations activate phospholipase A_2.[104] Arachidonic acid, prostaglandins and leukotrienes are produced when phospholipase A_2 hydrolyzes the cell membrane. Calcium-independent phospholipase A_2 produces reactive nitrogen species, MAPK, and ROS.[104] ROS cause the release of glutamate via the Transient receptor potential vanilloid 1 (TRPV1) and TRPA1 channels which are also involved with neuropathic pain.[104, 113] Glutamate release increases local glutamate receptors and

ion channels which in turn increases the release of cytokines and ROS and activation of glial cells and kinase cascades leading to pain.[89]

1.10. Autoantibody mediated pain

Autoantibodies to CNS proteins can elicit pain via multiple mechanisms. Complement binding of the Fc region of autoantibodies can produce inflammation, when this inflammation causes nerve damage neuropathic pain can occur.[45, 57, 117] For instance, complement-induced response ultimately leads to neuronal damage and can cause neuropathic pain as is found in Guillain-Barre syndrome.[57] Autoantibodies binding to nociceptors may damage the nerve cell or change its function resulting in neuropathic pain.[57] Sensitized nociceptors may become hypersensitive to noxious and/or non-noxious stimuli or become spontaneously active. Neuropathic pain mediated through Fab-region binding can have multiple effects by blocking the binding sites, causing a conformational change, or activating the antigen.[57] The binding of the antigen by the antibody may potentiate the inflammatory response and/or prevent the glial scar from forming properly perpetuating a permeable BSCB and neuropathic pain.[25]

Autoantibodies produced after SCI could work through any of these means and result in the development of neuropathic pain. Currently, there are no diagnostic tools available to predict who will develop chronic neuropathic pain after SCI. Therefore, this research sought to identify autoantibodies after SCI that may predict the development of neuropathic pain.

1.11. Knowledge Gaps.

Although both experimental and clinical studies have observed immune responses after SCI, it has not been investigated if human SCI elicits an autoantibody response. As a

part of this thesis research the following questions were investigated in human SCI subjects:

1. Does human SCI elicit an autoantibody response?
2. What is the antigen(s) that triggers autoantibody production?
3. Does the circulating level of identified autoantibodies correlate with the development or presence of neuropathic pain?

1.12. Study Impact

The identification of autoantibodies produced after SCI may lead to new treatment targets or new prognostic indicators.

Hypothesis: proteins from the injured spinal cord released by SCI trigger autoantibody production that can lead to the development of neuropathic pain.

Aim 1: To test whether SCI elicits an autoantibody response and identify the antigen(s) that triggers this response in human SCI subjects**.**

Aim 2: To statistically compare whether the presence of autoantibodies to specific antigen(s) correlates with the development of neuropathic pain in human SCI subjects.

Chapter 2. General Methods

2.1. Reagents, antibodies and proteins

Methods Table 1. List of reagents or kits

Reagent or Kit	Provider	Catalog Number
CDP-Star Reagent for alkaline phosphatase	Biolabs, Ipswich, MA	C0712
Albumin from Bovine Serum	Sigma Aldrich	A7906-100G and A3059-50G
microBCA assay kit	Thermo Scientific, Rockford, IL	23235
Melon Gel IgG Spin Purification kit (Antibody purification)	Thermo Scientific, Rockford, IL	45206
Pierce Protein A IgG Plus Orientation (protein A affinity columns)	Thermo Scientific, Rockford, IL	44893
Complement C3 ELISA	abcam	ab108822
Complement C5 ELISA	abcam	ab125963
Precision Plus Protein Standards	BioRad	161-0363

Methods Table 2. Antibodies used, provider and catalog number.

Antibody	**Provider**	**Catalog Number**
Primary Antibodies		
Custom rabbit anti-GFAP antibody	Custom – Dash lab	NA
Anti-CRMP2 (Rabbit)	Sigma Aldrich	C2993
Anti-CRMP2 antibody(Rabbit)	abcam	Ab36201 Rb, Lot GR212945-1
Anti-CRMP2 antibody (Rabbit)	ThermoFisher Scientific	PA5-29728
Secondary Antibodies		
Goat anti-human IgG (H+L) secondary antibody, Alexa Fluor 488 conjugate	Invitrogen by Thermo Fisher Scientific	A-11013
Goat anti-human IgG (H+L) seconday antibody, Alexa Fluor 568 conjugate	Invitrogen by Thermo Fisher Scientific	A-21090
Alkaline phosphatase goat anti-rabbit IgG antibody	Vector	AP-1000 anti-R, Lot YO222
HRP goat anti-human IgG antibody (peroxidase)	Vector	PI-3000, Lot ZA0709
Pierce Antibody Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase	Pierce Thermo Scientific	Prod #31460, Lot OG 1886-49
Anti-rabbit horseradish peroxidase secondary antibody for Wes	Protein Simple	DM-001, Lot number 26571

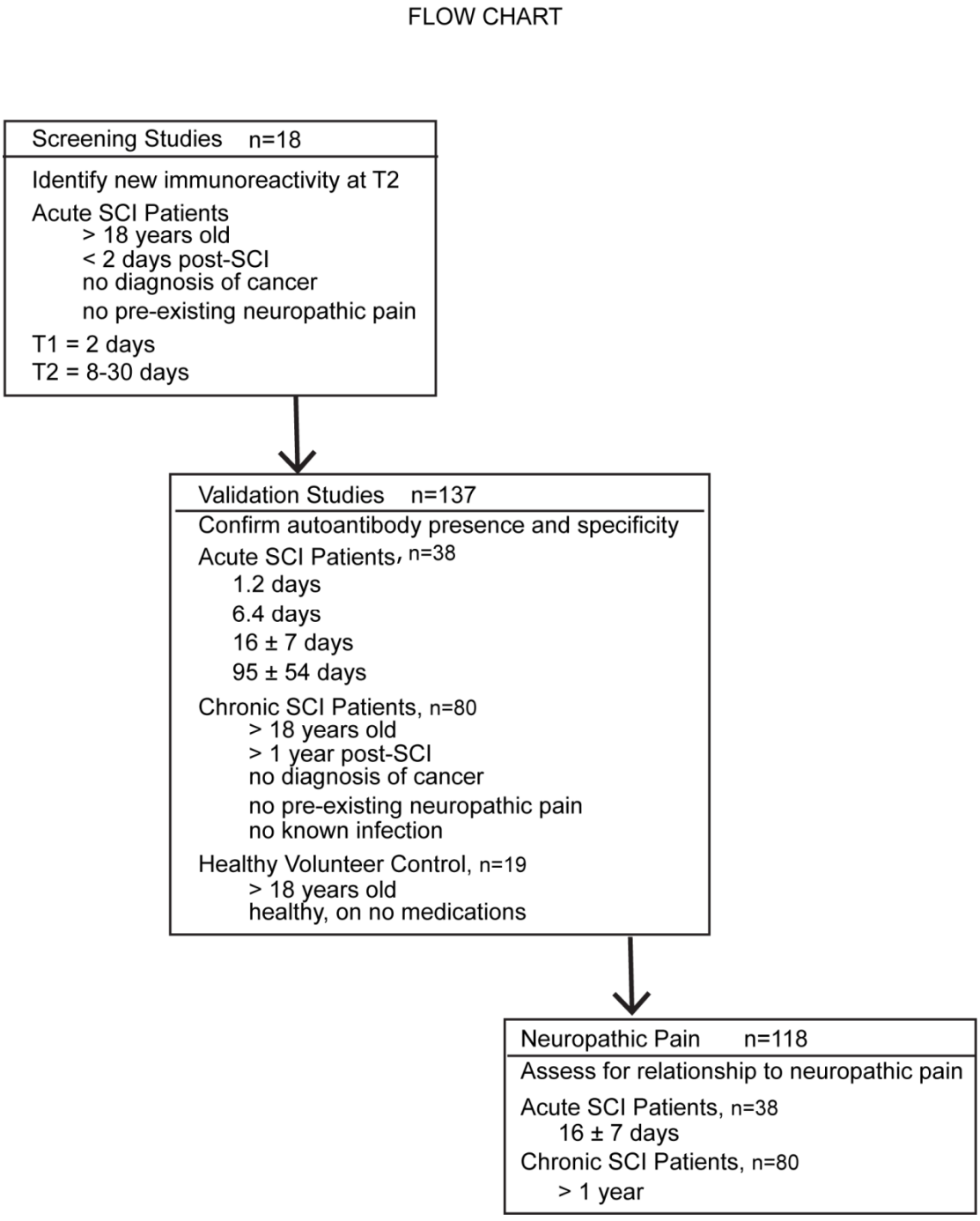
Methods Table 3. Purified Recombinant Proteins, Provider and Catalog number

Protein	**Provider**	**Catalog Number**	**Predicted Molecular Weight (kDa)**	**Reference peptide sequence**
Purified recombinant protein of human glial fibrillary acidic protein (GFAP), transcript variant 1	OriGene	TP304548	49.7	NP_002046
Purified recombinant protein of homo sapiens dihydropyrimidinase-like 2 (DPYSL2) (CRMP2)	OriGene	TP309080	62.1	NP_001377
Recombinant protein of human calreticulin (CALR)	OriGene	TP303222	46.4	NP_004334

2.2. Study Subjects

The protocol for the use of adult human subjects was reviewed and approved by the University of Texas Committee for the Protection of Human Subjects. All SCI subjects enrolled were over 18 years of age and had a traumatic, non-penetrating SCI with a neurologic deficit. Acute SCI subjects were enrolled within 2 days of SCI. Chronic SCI subjects were enrolled at greater than one year post-injury. Subjects were excluded if they had a known medical condition that accounted for neuropathic pain (e.g., diabetic neuropathy, renal insufficiency, HIV-associated, ethanol-associated neuropathy) or diagnosis of cancer within the previous 5 years. In addition, chronic SCI subjects were excluded if they had a known infection within 30 days of blood sampling. Blood samples were obtained after informed consent and were de-identified to provide confidentiality. Plasma from healthy volunteers was used as reference controls. Plasma from these groups of patients was used for autoantibody screening, validation and association with neuropathic pain studies (Figure 1). Demographic and clinical data were collected. Patients' neurological levels of injury were classified according to the ISNCSCI Scale.[30]

Figure 1. Flow chart of plasma used for autoantibody screening, validation and association with neuropathic pain studies.



Flow chart of subjects and samples used for autoantibody screening; T1= Time 1 (within 2 days after SCI); T2=Time 2 (8-30 days after SCI).

A. ISNCSCI

The ISNCSCI is a standardized method of classifying sensory and motor impairment in spinal injury for research and clinical practice.[30] Subjects enrolled in this study were ISNCSCI A-D defined as,

"A = Complete. No sensory or motor function is preserved in the sacral segments S4-S5.

B = Sensory Incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5 AND no motor function is preserved more than three levels below the motor level on either side of the body.

C = Motor Incomplete. Motor function is preserved below the neurological level, and more than half of key muscle functions below the single neurological level of injury have a muscle grade less than 3.

D = Incomplete. Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade greater than or equal to 3."[30]

American Spinal Injury Association: International Standards for Neurological Classification of Spinal Cord Injury

B. Classification of Neuropathic Pain

The definition of neuropathic pain was based on the clinical diagnosis and documentation of neuropathic pain. In order for a subject to be classified as having neuropathic pain there must have been documentation of pain in the medical record which included the descriptor *neuropathic*. The IASP has proposed a uniform classification system to define pain: nociceptive (musculoskeletal and visceral) and neuropathic (at level and below level of injury).[118] In accordance with the IASP and other recommendations, pain documented as "acute pain due to trauma" was not considered neuropathic pain nor

was nociceptive pain described as musculoskeletal, visceral or headache considered neuropathic.[119] Treatment with gabapentin or pregabalin, commonly used medications for neuropathic pain, was used as confirmation of the neuropathic categorization. All of the chronic and 18 of the acute subjects completed the Leeds assessment of neuropathic symptoms and signs (S-LANSS) pain scale and the Short-Form McGill Pain Questionnaire as additional validation of their pain classification.[120-122] The S-LANSS scale has been validated against clinical judgement.[121] These scores were corroborated with the clinician's assessment of pain. The S-LANSS self-assessment form was administered in a standardized manner. Subjects were instructed to consider their pain within the last week and to focus on the most severe pain. The instrument includes a numeric visual analog scale for rating pain intensity and diagram on which the subject indicates where the neuropathic pain exists. Descriptors on the S-LANSS and in the McGill Pain Questionnaire include words commonly associated with neuropathic pain (e.g., pins and needles, electric shock, burning). The pain onset, duration, aggravating or mitigating factors and whether or not the patient had spasticity was also documented.

2.3. Plasma Samples

For the initial screening for autoantibodies, blood samples were collected in EDTA tubes (BD, Franklin Lakes, NJ USA) from 18 adult, acute, traumatic SCI patients at two time points: 1) within 2 days of injury (Time 1, T1); and, 2) within 8-30 days of injury (Time 2, T2). A study has demonstrated that IgG titers peaked 14-33 days after brain injury [123], therefore, it was anticipated that autoantibodies related to injury would not be present close to time of injury and would emerge within the Time 2 period. Blood cells were removed by centrifugation (4°C, 800 X g for 10 minutes), plasma was collected, and platelet-poor plasma prepared by centrifugation (4°C, 10,000 X g) for 10 min. Samples were aliquoted and frozen at -80°C until assayed.

To confirm the presence of autoantibodies to a specific CNS protein, plasma was collected and processed in the same manner as described above for acute and chronic SCI patients and healthy volunteers. Autoantibody confirmatory studies were performed on plasma obtained from 38 adult, acute, traumatic SCI patients at four time points post-injury: 1.2 ± 0.7, 6.4 ± 1, 16 ± 7 and 96 ± 54 days post-injury. Thirteen of these subjects provided the sample at 96 ± 54 days post-SCI. The 80 chronic SCI subjects (> 1 year post-SCI) and 20 healthy volunteers provided a one-time blood sample.

2.4. Preparation of Human Cadaver CNS Homogenate

It is not possible or humane to obtain CNS tissue samples from individual patients to test for IgG reactivity to self-proteins. For the purpose of this study, human cadaver CNS tissue was used as a substitute for the patient's own tissue and antibodies produced in response to this tissue were classified as autoantibodies. Human cadaver CNS tissue samples (from one individual who died from complications of Alzheimer's disease (AD)) and three individuals without confirmed AD were obtained from the UT-Health Willed Body Program and were frozen at -80℃ until ready for use. The mean time from time of death to tissue extraction was 16 hours. Cadaver tissue was collected from an 1) elderly male with a medical history of AD, cluster headaches, and concussion in 1965 after being hit by a car while riding a bike. 2) A 75 year old female with possible history of dementia, post-mortem interval from time of death until tissue extraction was less than 24hrs. 3) A 75yo male with history of cardiac bypass, post-mortem interval was approximately 14 hours until tissue procurement; and 4) A 67 year old female with post-mortem interval of 12 hours. The subject appeared to be of low to normal body weight, no medical history was provided.

CNS tissue from the four cadavers listed above was used for 1- and 2-Dimensional (1-D and 2-D) gel studies. The rationale for use of multiple sources of CNS tissue was to assure that anti-mortem factors from any one cadaver and/or post-mortem

time did not dictate study results.[124] For instance, glial cells and specifically, astrocytes have been associated with Alzheimer's disease (AD). [125] Post-translational changes, including glycosylation, phosphorylation and increased GFAP expression have been reported in the AD brain. [126] Increased CRMP2 phosphorylation is present in the AD brain.[127] As the initial cadaver donor had AD, to assure that reactivity to the CNS homogenate was not specific to post-translational changes with one specific homogenate donor, testing was performed using different donors who did not have AD. Experiments on both 1-D and 2-D gels were repeated with brain homogenate from 4 different cadaver donors, only one of which had a reported clinical diagnosis of AD. The latter 3 cadaver brains were perfused with PBS prior to CNS tissue procurement. Cadaver CNS tissue was snap frozen in liquid nitrogen which causes rapid, temporary inactivation of all reactions that occur in extracted tissue.[128] Neural tissue is sensitive to protein degradation (for instance degradation fragments) which could impact CRMP2 or GFAP proteins; however, no degradation was seen in post-mortem interval analysis of GFAP.[124, 128]

CNS tissue samples were homogenized in a buffer consisting of radioimmunoprecipitation assay buffer (RIPA) containing protease (1mM PMSF in DMSO; 10 µg/ml leupeptin hemisulfate) and phosphatase (1 mM NaF; 0.2 mM Na pyrophosphate, 100 nM okadaic acid in DMSO). Protein concentration was estimated using a microBCA assay kit (Thermo Scientific) and tested on western blot as described below. Results from samples from spine tissue were replicated in brain tissue. Because brain tissue was more accessible the western blot experiments discussed below were conducted using brain tissue homogenates (total human CNS protein).

2.5. Protein quantitation

A microBCA assay kit (Thermo Scientific) was used to quantify protein concentrations in CNS protein/brain homogenate samples. Bicinchoninic acid (BCA) was used as the detection reagent of Cu++. Cu++ is formed when Cu2+ is reduced by protein in an alkaline setting. A standard curve with 6 serial dilutions was made in duplicate using bovine serum albumin (BSA). Two hundred microliters of the protein assay mix of reagents MA, MB, MC (combined in the ratio of 25:24:1) were applied to each well. Standards, plain buffer, and unknowns were applied to a 96 well plate and allowed to incubate for 45 minutes. The results were read on a plate reader at 562nm. Secondary measurements were made with a Beckman coulter spectrophotometer set at 280nm wavelength.

2.6. Western Blot

Preliminary western blot studies were performed on a subset of plasma samples. Precision plus protein standards (BioRad) were used as molecular weight standard markers. Total human CNS protein (10 µg) was resolved on 4-12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex, Life Technologies, USA) and transferred to Immobilon-P membrane (Millipore). Membranes were blocked overnight at 4℃ in a Tris-buffered saline solution containing 1% Tween-20 (TBST), 5% bovine serum albumin (BSA) and 1%milk. Blots were incubated for one hour at room temperature in primary antibody (human plasma (1:1000) or rabbit anti-GFAP (1:6000) or rabbit anti-CRMP2 (1:1000)) that was diluted in a 5%BSA, 1%milk solution (1:6000), washed with TBST, incubated for one hour at room temperature in secondary antibody (anti-human or anti-rabbit alkaline phosphatase antibody 1:20,000 (Vector) as appropriate in TBST containing 2%BSA and 1%milk), and again washed in TBST. Membranes were developed with CDP-Star Reagent for alkaline phosphatase (BioLabs, Ipswich, MA).

Membranes were tested with and without primary antibody to distinguish between the primary and secondary antibodies immunoreactivity. Plasma samples from subjects that were determined to be positive or negative on the initial western blot screening were re-screened to confirm immunoreactivity patterns using both CNS homogenate and purified human recombinant GFAP (GFAP, OriGene TP304548) or purified human recombinant CRMP2 (CRMP2, OriGene TP309080). A custom-made anti-GFAP antibody or a commercial anti-CRMP2 antibody was used as a positive control.

2.7. 2-Dimensional gel Electrophoresis

The first sets of 2-D gels were performed with Immobiline DryStrip gels, pH 3-11(immobilized pH gradient, IPG) (GE Healthcare Life Sciences, Pittsburgh, PA). For the first dimension of the gel the IPG gels were rehydrated overnight. Enough CNS protein sample was prepared for 135 µl per IPG gel. This consisted of 50µl of 4µg/ul (200µg) CNS homogenate, 80µl Destreak buffer (GE Healthcare Life Sciences; containing urea, thiorurea, CHAPS and Destreak reagent), and 1.3µl (1%) ampholytes. Each of 4 lanes of the gel hydration chamber was loaded with 125µl of CNS protein in buffer. The IPG strip was carefully placed on top of the sample assuring to keep the sample under the strip and avoid trapping any air. About 3ml of mineral oil cover fluid was applied on top of the IPG and assuring to place cover fluid at each end of the IPG to keep the sample under the gel. Then the IPG gels and samples were covered and allowed to rehydrate at room temperature overnight (Figure 2A).

The IPG gels were rinsed with deionized water and placed in the IEF chambers and covered with oil, again allowing no air bubbles. The IEF chambers containing the IPG gels were positioned so that the positive end (acidic) of the IPG strip was placed toward the anodic electrode (+) and the negative end of the strip was placed toward the cathodic electrode (-) (Figure 2B). The top was placed on the Ettan IPGphor II machine and a step

gradient step protocol was run (1: 300v for 1hr, 300vhr at 15℃; 2: 1000v for 0:46hr, 500vhr; 3: 1000v for 0:30 hr, 500vhr; 4: 5000v for 1 hr; 5: 500 v for 1 hr; 6 100v for 11 hr, 1100vhr) (Figure 2C). Starting with a low voltage reduces protein aggregation. During the isoelectric focusing the blue dye migrates toward the anode, Figure 2D shows the dye has cleared.

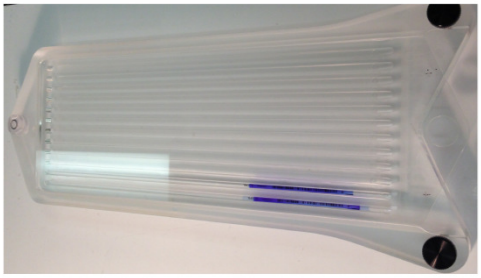
Once the step gradient protocol was completed, the IPG strips were rinsed briefly in deionized water and placed in 15ml conical tubes containing equilibration buffer (6M urea, 30% glycerol, 2%SDS, 1M Tris(pH6.8), 65mM DTT, and bromophenol blue as a tracking dye) with the first tube including DTT reagent (0.14g/14ml) as a reducing agent for 30 minutes on a rocker followed by a second tube with Iodoacetamide 2.5% (0.35g/14ml equilibration buffer) to fix and equilibrate the sample (30 min on a rocker). Following this the IPG gel was placed in the sample well on an 8% acrylamide gel (with the positive end/low *pI* proteins on the side near the molecular weight markers). The second dimension separation was performed with NuPAGE MES SDS running buffer and MES SDS (Figure 2E).

Two gels were placed in GelCode Blue for 1-8 hours then stored in 10% acetic acid. Two gels were transferred to Immobilon-P membranes using NuPage transfer buffer with 15% methanol. Transfer was started at 30V then run for 90 minutes at 150mAmps. After transfer membranes were blocked with 5% BSA and 1% milk for 12 hours on a rocker. Membranes were then incubated with purified IgG from Time 1 (T1) or Time 2 (T2) plasma in 5%BSA, 1% milk (1:500) for 3 hours. After rinsing in TBST (5 times), membranes were incubated for 1hr in alkaline phosphatase secondary antibody in 2% BSA, 1% milk (1:20,000). Membranes were rinsed again and placed in CDP-Star reagent buffer and developed. The film was marked to assure proper alignment of the membrane-generated spots with spots on the GelCode blue stained gel. New spots found in T2 were identified and extracted from the corresponding gel for protein identification.

Figure 2. 2-Dimensional Gel Electrophoresis

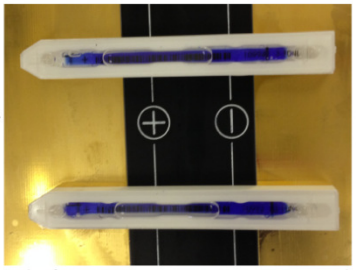
First Dimension Isoelectric Focusing (IEF) Separation

A



IPG Strip Rehydration

B



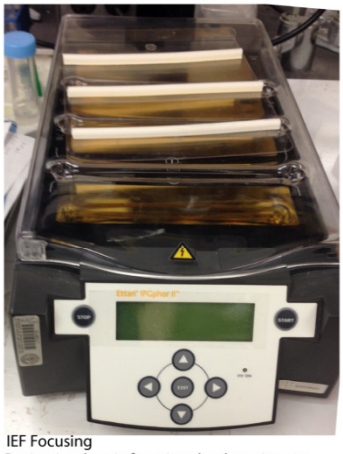
Anodic electrode area (+)

Cathodic electrode area (-)

Positive side of IPG strip (Low pI proteins)

Negative side of the strip (High pI proteins)

C



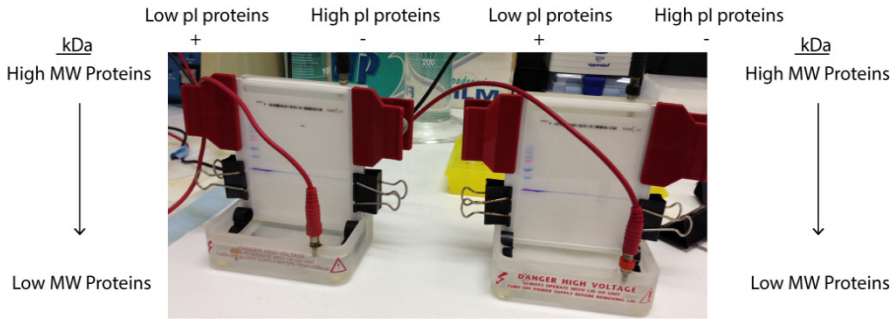
IEF Focusing
During isoelectric focusing the dye migrates toward the anode (+)

D

IEF Focusing is complete, the sample is now ready for the second-dimension separation

Second Dimension Separation

E



2.8. Large Scale 2-Dimension Gel Electrophoresis

Large scale 2-D gels (20 x 22 cm) were used in order to spread out immunoreactive spots and improve the precision of spot selection for protein identification using liquid chromatography-mass spectrometry (LC-MS/MS). Large scale 2-D gels were prepared in duplicate, one for Coomassie staining and one for western analysis according to a protocol provided by Kendrick Laboratories (Madison, WI). [129, 130] Brain homogenate containing phosphatase and protease inhibitors and SDS boiling buffer was treated with Omnicleave and heated in a boiling water bath for 5 minutes. Protein concentration was ascertained using the BCA Assay. Two-dimensional electrophoresis was performed using the carrier ampholine method of isoelectric focusing. [129, 130] Brain sample (250µg) was loaded on the first dimension gel. Each sample included an internal standard (tropomyosin (33,000)) and molecular weight markers (myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000) (Sigma Chemical Co., St. Louis, MO and EMD Millipore, Billerica, MA) as reference points for orientation for later spot identification on the Coomassie stained 10% acrylamide gels (second dimension gel). SDS second dimension gel electrophoresis was carried out for 5 hours at 25 mA/gel. Duplicate gels were then transferred onto PVDF membranes using the same molecular marker proteins as reference points.

To identify cross-reacting self-antigens, sections from two Coomassie-stained sister 2-D membranes between approximately 30-75 kDa were cut and imaged. Then the membranes were blocked, and probed with T1 or T2 plasma from samples found to produce new immunoreactivity using T2 plasma on screening western blots. Concentrating on the 30-75 kDa section of the 2-D membrane allowed conservation of precious plasma sample. Protein spots on the 2-D T2 membrane with new or enhanced immunoreactivity were identified. The scanned membrane images, tropomyosin marker and standards were used as guides, and calipers were used to measure the precise locations of areas of

enhanced T2 membrane immunoreactivity. Clear transparency film was placed on top of the membrane and the Coomassie-stained companion gel. The spots were marked on the membrane transparency and this was matched to the gel and identified spots were circled and numbered. Prior to excising spots, the locations were validated with the caliper measurements. A photograph was taken of the numbered spots. The gel was wiped with 100% methanol and the spots were carefully cut out around the edges using a sharp, fresh scalpel. Samples were speared with the tip of the scalpel and placed in sterile Eppendorph tubes and capped immediately. Next 200µl of ultrapure water per spot was added to the Eppendorph tubes. This was allowed to sit for 20 minutes prior to vortexing. The vortexing removed the plastic film from the gel. The water was aspirated and the gel was moved to a fresh Eppendorph tube labeled with the spot number. These spots were sent for LC-MS/MS identification.

2.9. LC-MS/MS and protein identification

LC-MS/MS was performed by the Darie Laboratory at Clarkson University according to published protocols.[131, 132] In brief, gel spots were washed rehydrated and trypsin digested. The peptide mixture was analyzed by reversed phase liquid chromatography (LC) and MS (LC-MS/MS) using a NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Ultima API MS (Micromass/Waters, Milford, MA), as previously described.[131-135] A Mascot and PLGS database search provided a list of proteins for each gel spot. The MS/MS spectra for the proteins identified by either one peptide or a Mascot score lower than 25 were verified to eliminate false positives.

2.10. Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). Descriptive data for subject groups was summarized as means and standard deviations

(SD). Data was tested for normality using the Kolmogoroff-Smirnov test. For data that did not pass the normality test, the Mann-Whitney Rank-Sum Test was used and the data are presented as medians ± SE. Kruskal-Wallis One Way Repeated Measures ANOVA followed by Dunn's Method for multiple comparisons was used when comparing the three time points for acute SCI data. Spearman Rank Order Correlation was used to assess for correlation between the continuous non-parametric GFAPab immunoreactivity or CRMP2ab immunoreactivity and plasma complement C3 and C5 levels. Two Way Repeated Measures ANOVA followed by Holm-Sidak method for all pairwise multiple comparison was used for comparing the C3 and C5 levels over time by pain group. Chi-square test was performed to determine the relationship between the presence of multiple autoantibodies and the development of neuropathic pain. Multiple logistic regression was performed to assess the relationship between the presence of multiple autoantibodies and neuropathic pain while controlling for other variables. Significance was defined as $p \leq 0.05$.

2.11. Method to Screen for autoantibody detection

Figure 3. Flow chart of methods for autoantibody detection

A. Patient samples used for autoantibody screening.

Patients were enrolled prospectively and classified according to their level of injury and presence or absence of neuropathic pain (pain group). The first autoantibody screening experiments (Figure 3) performed were on the first consecutive 18 acute SCI subjects enrolled to determine whether new immunoreactivity could be identified

subsequent to SCI (Table 4). It is not possible to collect patient spinal cord tissue, nor is it possible to test pre-injury samples from human SCI subjects to establish a baseline. Therefore, a baseline sample <48 hours following injury (T1) served as the comparator to a sample obtained 8-30 days post-injury (T2). Demographics on these acute SCI subjects are presented in Table 4. Subjects were primarily male (94%) with an average age of 38.1±14.6 years, 78% had complete or sensory incomplete injury (ISNCSCI A or B) and 78% were cervical neurological level injuries.

Table 4. Demographics of Subjects Used in Screening Studies.

Subject No. (n=18)	Age	Gender	Mechanism of Injury	ISNCSCI	Neurological Level of Injury
1	61	Male	Bicycle	A	C4
2	37	Male	Motor vehicle collision	A	T3
3	37	Male	Fall	C	T12
4	26	Male	Fall	C	C5
5	20	Male	Motor vehicle collision	A	T6
6	36	Female	Motor vehicle collision	A	C4
7	18	Male	Motor vehicle collision	A	C7
8	57	Male	Motor vehicle collision	A	C5
9	69	Male	Fall	C	C5
10	39	Male	Bicycle	A	C4
11	26	Male	Diving	A	C6
12	21	Male	Motor vehicle collision	B	T9
13	30	Male	Motor vehicle collision	A	C5
14	52	Male	Assault	A	C4
15	35	Male	Car fell on his neck	C	C2
16	48	Male	Motor vehicle collision	A	C3
17	30	Male	Motor vehicle collision	B	C5
18	44	Male	Motor vehicle collision	A	C4

B. Western blots for autoantibody screening

Western blots for autoantibody screening were performed as described in Chapter 2.7. Total CNS protein homogenate (10 µg) was separated on 4-12% SDS-PAGE gels and transferred to Immobilon-P membranes. Western blots were performed on samples from 18 subjects at T1 and T2 to identify new immunoreactivity at T2 that was not present in T1. These results of these western blots were dichotomized as positive or negative for new immunoreactivity based on visual inspection. Additionally, similarities in molecular weight of new immunoreactive bands between different patients' T2 plasma samples were assessed. A pattern of new immunoreactive bands ranging in size between 35-50kDa was found in 4/18 (22%) subjects (Figure 4).[136]

Figure 4. Representative western blot probed with human SCI patients' plasma obtained at T1 and T2.

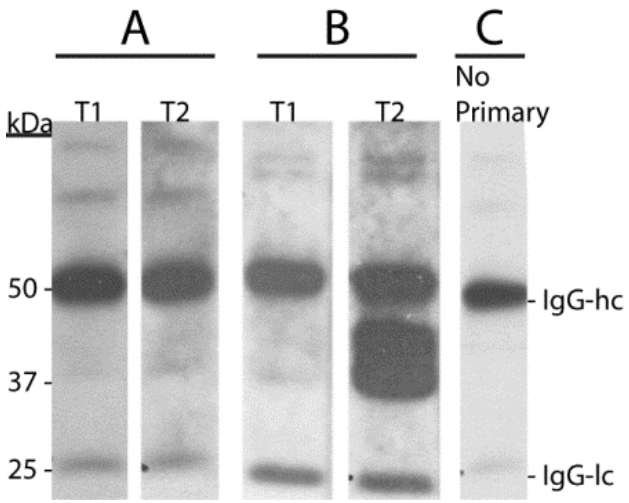
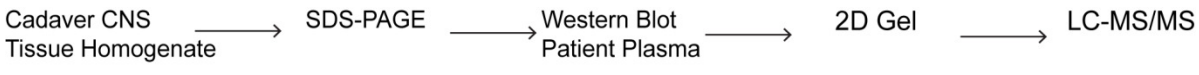


Figure 4. Representative western blot probed with human SCI patients' plasma obtained at T1 and T2. The protein source for the gel was human cadaver CNS homogenate. To reduce IgG contribution from the donor cadaver, the cadaver was perfused with PBS prior to tissue extraction. A) a subject without change in immunoreactivity between T1 and T2, B) a subject with increased immunoreactivity between 35-50kDa at T2 compared to T1, and C) immunoreactive patterns resulting from IgG present in the cadaver homogenate, present when no primary was used. Bands at 50 and 25kDa are heavy chain (hc) and light chain (lc) immunoglobulin G (IgG). The new immunoreactivity between 35-50kDa was present in 4 of 18 subjects tested.

2.12 Method to identify antigens for autoantibodies

Figure 5. Flow diagram of experiments conducted to identify target antigens



Multiple techniques were tried to isolate the protein antigen. For instance, IgG purification was performed and affinity columns were created using recombinant protein A to covalently crosslink the antibody in the plasma sample. CNS homogenate was incubated in the column and glycine elution was performed (Appendix). The amount of antigen eluted from the column was found to be insufficient and, therefore, a new approach, 2-D gel electrophoresis was tested (Figure 5).

A. 2-D gels electrophoresis to separate human cadaver CNS protein

2-D gel electrophoresis as described in section 2.7 was used to separate human cadaver CNS protein homogenate by isoelectric focusing and molecular weight. 2-D gels were run in duplicate, one for each western blotting and Coomassie staining. Membranes from the 2-D gels were probed with T1 or T2 SCI subject plasma known positive for new immunoreactivity at T2. This was repeated using 3 different cadaver donors and two different SCI subjects' plasma. New or enhanced immunoreactivity was found at T2 in the area between 35-70kDa. (Figure 6).

Figure 6. 2-D Gel with human CNS protein and membranes probed with SCI patients' plasma

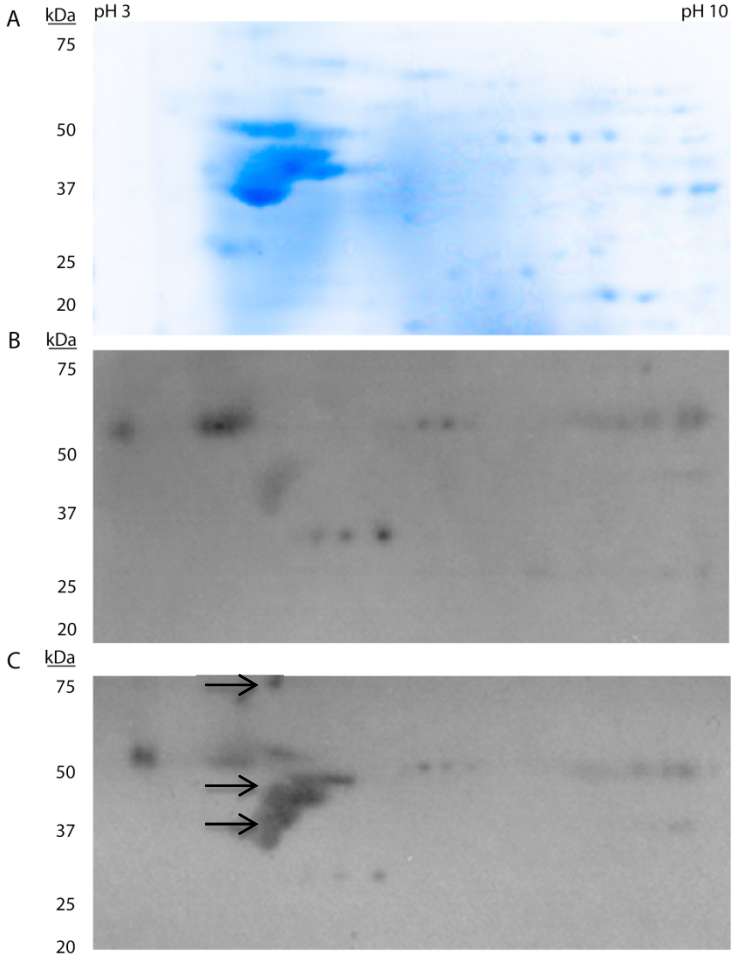


Figure 6. 2-D gel electrophoresis was used to separate human cadaver CNS homogenate by isoelectric point (*pI*) and molecular weight. A) GelCode Blue stained gel showing spots which are proteins that have been separated by *pI* and molecular weight. B and C) Membranes derived from a corresponding 2-D gels; B was probed with T1 SCI patient plasma, C was probed with T2 SCI patient plasma. This SCI subject was known to be positive for new immunoreactivity in the 37-50kDa range at T2. New immunoreactivity can be seen in membrane C (arrows).

B. Excision of protein spots from 2-D gels

Protein spots of interest were identified using 2-D gels and western blot analysis and excised as described in Methods 2.8. Large 2-D gels and corresponding membranes were prepared in order to further separate the spots. Numerous membranes were probed with two SCI patients' T1 and T2 plasma. Areas of enhanced immunoreactivity at T2 were identified. Spots showing new immunoreactivity were carefully excised from the corresponding Coomassie-stained 2-D gels and prepared for analysis by LC-MS/MS. An example of membranes from a SCI subject's T1 and T2 plasma and locations of spots excised from the Coomassie-stained 2-D gel is shown in Figure 7.

Figure 7. Large scale 2-D membranes and Coomassie stained gel showing spots selected for LC-MS/MS.

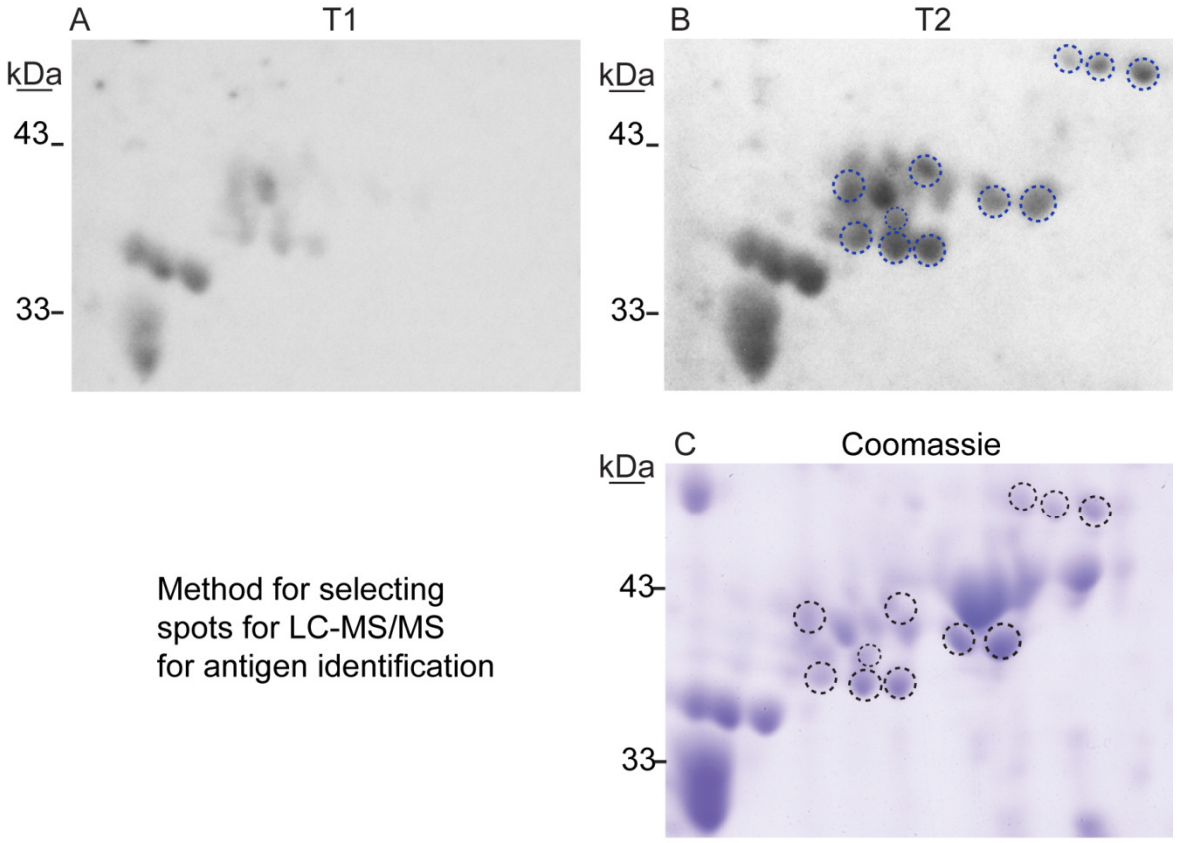


Figure 7. Large scale 2-D membranes and Coomassie stained gel showing spots selected for LC-MS/MS.

2-D gel membranes probed with T1 (A) and T2 (B) patient plasma showing enhanced immunoreactivity at T2 compared to T1 from one of the four patients that previously demonstrated increased immunoreactivity at T2 on 1-D gel membranes. Spots circled in blue were identified for sequencing. They were located and cut out of the corresponding Coomassie stained gel (C) and sent for identification by LC-MS/MS. Glial fibrillary acidic protein (GFAP) and collapsin response mediator protein 2 (CRMP2) were the predominant proteins identified.

C. LC-MS/MS protein identification

Excised proteins were subjected to LC-MS/MS and identified as described in Chapter 2.9.

Dihydropyrimidinase-related protein 2 isoform 2 also known as collapsin response mediator protein 2 (CRMP2) and glial fibrillary acidic protein (GFAP) and were the most commonly identified proteins on the spots analyzed. CRMP2 was found in 11 spots and GFAP was found in 7 spots when probed with different SCI patients' plasma. The mass spectrometry results for these proteins are presented in Table 5. The Mascot score provided in Table 5 column three is the calculated probability (P) that the peptide match between the experimental data and the database sequence is a random event (reported as -10Log(P)). Peptides found above a pre-defined significance threshold are separated from random scores. Generally, a higher Mascot Score is a higher probability of a correct match; the score is separated out from the distribution of random scores indicating the match is not a random event. All of the reported identified peptides have matches above the significance threshold. As noted in section 2.9, Mascot scores lower than 25 were verified to eliminate false positives. Of 22 spots evaluated there were 886 unique peptides mapped; 490/886 (55%) and 175/886 (19.8%) showed peptides that mapped to CRMP2 or GFAP respectively. The remaining peptides may have resulted from contamination of nearby proteins or they could be additional candidates. For example, a minority of peptides that mapped to β-actin were present in two spots. However, when the blot was re-probed with antibodies specific to β-actin, the immunoreactive signal did not co-localize with the location of these spots, but rather an abundant adjacent spot. Multiple GFAP isoforms and their post-translational modifications and have been shown in a staircase-like pattern in brain tissue similar to what is found on the 2-D gels.[126, 137] CRMP2 protein and post-translational modifications have also been seen with multiple spots in other studies as CRMP2 is a highly phosphorylated protein.[138]

Table 5. LC-MS/MS Findings

Number of Spots identified	Protein Identified	Ranges of Mascot Scores	Range of Number of unique peptides found per spot	NCBI Accession Number (gi number GenInfo Identifier)
11	dihydropyrimidinase-related protein 2 isoform 2 [Homo sapiens]	50 - 1005	9-109	gil4503377
7	glial fibrillary acidic protein isoform 1 [Homo sapiens]	142-1362	8-41	gil4503979

Chapter 3. Human SCI increases autoantibodies to CRMP2

3.1. Introduction

A. Collapsin Response Mediator Protein 2 (CRMP2)

The cytosolic protein collapsin response mediator protein 2 (CRMP2) is also known as dihydropyrimidinase-related protein 2 (DPYSL2, DRP-2) or turned-on-after-division-64 (TOAD-64).[139] CRMP2 is a member of the CRMP family of phosphoproteins. All of the CRMPs (1, 2, 3, 4 and 5) are highly expressed in development with CRMP2 being the most abundant CRMP at maturity.[139] In adults CRMP2 is expressed in neurons and oligodendrocytes in the spinal cord and brain, and it has also been found in monocytes and lymphocytes.[138, 140] CRMP2 has multiple known binding partners with which it affects neurite growth, polarity, guidance, neurotransmitter release and calcium balance.[139] CRMP2 activity is regulated through kinase-induced phosphorylation. CRMP2's phosphorylation by cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase-3β (GSK3β) is necessary for the semaphorin 3A (sema3A)-induced growth cone collapse which operates through microtubule reorganization or destabilization.[141, 142]

Along with sema3A, CRMP2 participates in axon guidance exerting an inhibitor role. Sema3A is an axonal growth inhibitor which is expressed in fibroblasts and glia in the glial scar 1 day to 4 weeks after SCI.[143] Kaneko et al. used a specific sema3A inhibitor derived from fungus (SM-216289) in spinally transected rats to determine whether neutralizing sema3A permits axon regrowth after SCI. SM-216289 inhibits growth cone collapse and supports neurite lengthening.[143] The sema3A inhibitor was delivered to rats via an osmotic mini-pump for 4 weeks after transection. More axonal regeneration

within the lesion site in the sema3A inhibitor treated rats was observed compared to control rats; some of these regenerated axons were from Schwann cells (PNS) that had migrated. Calcitonin gene related peptide (CGRP)-positive fibers were increased in the sema3A inhibited group compared to vehicle control group which was interpreted as sprouting of unmyelinated C fibers which are associated with pain. However, no allodynia was reported in these rats.[143] The effect of sema3A inhibition resulted in sprouting.

A separate study utilized a CRMP2-specific binding drug, Lanthionine ketamine (100mg/kg/d IP for 4 weeks) in a thoracic 7 level SCI incomplete transection mouse model. Fibrous scar tissue was measured by immunohistochemistry of Collagen IV fibrous tissue within the glial scar and axonal sprouting was detected using 5-HT. Lanthionine ketamine treated SCI mice had smaller areas of fibrous scar tissue and more axonal sprouting in the serotoninergic raphespinal tract than vehicle treated SCI mice.[144] The intent of both the sema3A inhibitor study and the Lanthionine ketamine study was to promote axonal regeneration through the glial scar; however CRMP2 and sema3A's effect glial scar formation, axon extension and axon guidance was also demonstrated. The glial scar is thought to be important for containing the injury site acutely after SCI and protecting intact tissue from damage. (Please see section 4.1.A for additional discussion on the glial scar.)

CRMP2 promotes neurite growth and modification of cell structure through interactions with tubulin, actin and neurofilaments.[139, 145] CRMP2 expression was co-localized with cytoskeletal protein βIII-tubulin in the white matter and marginal layer of the spinal cord in a study of developing chick spinal cords demonstrating CRMP2's involvement in axon elongation and cytoskeletal structure modification.[141] The expression patterns of CRMP2 on transverse sections of chick spinal cords were compared at developmental stages permissive for regeneration (E11) and non-permissive for regeneration (E15) in SCI crush injured versus sham operated chick spinal cords. After injury there was a change in CRMP2 expression from white matter to gray matter, but there

was not a difference in level of CRMP2 protein expression.[141] The CRMP2 redistribution demonstrated CRMP2 movement from axons to the cell body as a response to injury.

Cell culture studies of human T cells identified CRMP2 in T cells and determined that CRMP2 is involved with T cell migration and conformational changes.[140] In order for T cells to migrate they must first become polarized. During T cell polarization CRMP2 relocates to the trailing edge (uropod) of T cells with vimentin (an intermediate filament protein) where it is involved in motility through adhesive functions and cytoskeletal rearrangement.[140] Semaphorins are involved in lymphocyte migration by steering and facilitating or impeding cell motility.[140] CRMP2 is downstream of sema3A and transduces sema3A guidance signals in addition to CRMP2's participation in cell migration.[140] CRMP2 presence in the uropod depends on the activated status of the T cell. The presence of CRMP2 is important in directing T cells to the injury site. For example, blood from patients who were infected with retrovirus HTLV-1 associated with neuroinflammation had higher CRMP2 levels in activated T cells compared to healthy donor blood or HTLV-1 infected asymptomatic carriers.[140] Flow cytometry analysis of the HTLV-1 patients' peripheral blood mononuclear cells (PBMC) identified CRMP2 in CD4+ and CD8+ T cells and CD14+ monocytes/macrophages.[140]

CRMP2 phosphorylation is increased after SCI and increased phosphorylation of CRMP2 has been associated with poorer outcome.[141, 146] Chondroitin sulfate proteoglycans (CSPG) are also increased after SCI and CSPG inhibits axonal growth through GSK3β activation.[147] Inhibition of CRMP2 phosphorylation reduces CSPG-induced inhibition of axonal growth, improves microtubule stabilization and increases sensitivity to brain derived neurotrophic factor (BDNF).[146] BDNF is supportive of axonal growth. Nagai et al., using a near-complete dorsal transection SCI mouse model, observed an increase in pCRMP2T509 (CRMP2 phosphorylation site of GSK3β) after SCI in *CRMP2*+/+ mice, but CRMP2 protein expression had not changed. Phosphorylation at

T509 was inhibited in *CRMP2*KI/KI mice. The SCI *CRMP2*KI/KI mice had better locomotor recovery, regained nociceptive function and increased growth-associated protein 43 (GAP43) protein levels at the lesion site compared to *CRMP2*+/+ mice related to the CRMP2 phosphorylation inhibition provided by the knock-in model.[146] Dorsal horn CGRP-positive (nociceptive) and 5-HT-positive (locomotor circuitry) fibers were present at an increased density in *CRMP2*KI/KI mice and the inhibition of the phosphorylation of CRMP2 in these mice also resulted in less immunoreactivity to GFAP (suggesting a reduced inflammatory response) and a more compact glial scar.[146] These studies illustrate that CRMP2 and its phosphorylation impacts axon sprouting, the glial scar and inflammatory responses[146].

B. CRMP2 and neuropathic pain

Aberrant nociceptive fiber sprouting could lead to neuropathic pain.[148-150] Nerve growth factor (NGF) enhances nociception and induces axonal elongation and sprouting.[145, 151] A study that used dissociated sensory neurons from chick dorsal root ganglia demonstrated that neutralizing CRMP2 potentiated NGF-induced neurite outgrowth.[145] Investigators neutralized CRMP2 activity with anti-CRMP2, or with a dominant-negative form of CRMP2, in the presence of NGF which resulted in more DRG with neurites and lengthened neurites.[145] Additionally, CRMP2 antibodies triturated into DRG neurons blocked sema3A-induced growth cone collapse suggesting that CRMP2 is involved as a negative regulator of NGF-induced neurite growth.[145] Through inhibition of CRMP2, sprouting was induced; CRMP2 functionally inhibits sprouting.

NGF mRNA expression in adult rat spinal cord was shown to be elevated up to 4 days after injury indicating conditions for NGF-induced neurite growth after SCI.[152] CGRP immunoreactivity (nociceptive) is shown to increase in deeper lamina III and IV in spinally hemi-sectioned rats; however the administration of anti-NGF in this model reduced

CGRP expression in lamina I and II and prevented projection of CGRP into lamina III and IV suggesting anti-NGF inhibited sprouting of primary pain afferents.[151]

These studies support the possibility that after SCI an autoantibody to CRMP2 could contribute to blocking sema3A-induced growth cone collapse and/or increase the number of cells with neurites and the length of neurites in the DRG. These neurites, that would normally extend only to lamina I and II, in the presence of autoantibody to CRMP2 may project into deeper lamina. This sprouting of sensory neurons into deeper lamina related to the presence of an autoantibody to CRMP2 could lead to neuropathic pain.[150]

CRMP2 is a regulator of N-type voltage-gated calcium channel activity. The binding of CRMP2 to N-type voltage-gated calcium channels in a Cdk5-phosphorylation dependent manner amplifies calcium currents and increases neurotransmitter release in sensory neurons.[146, 153] Inhibition of this interaction or inhibition of the phosphorylation of CRMP2 reduces transmission of pain signals, possibly because of CRMP2's regulation of voltage-gated channels.[154] However, it is possible that an autoantibody to CRMP2 could interfere with the CRMP2-N-type voltage gated calcium channel regulation balance. The abnormal calcium influx into N-type voltage-gated calcium channels has been suggested to be related to neuronal excitability and pain.[154]

The hypothesis is that an autoantibody to CRMP2 contributes to the development of neuropathic pain. The potential for a relationship between the development of neuropathic pain and the presence of CRMP2ab in SCI patient plasma may result in clinically important findings. Therefore, correlative experiments with human plasma samples were performed to validate findings of CRMP2 as a potential antigen for the production of autoantibodies involved in the development of neuropathic pain.

3.2. Methods.

A. Fluorescent western blots on 2-D gel membranes

Fluorescent western blotting was performed to determine whether there are overlapping areas of immunoreactivity produced by T2 SCI patient plasma and a commercial anti-CRMP2 on 2-D gel membranes. A section of a 2-D gel membrane at the same molecular weight and isoelectric point range that was used to identify CRMP2 as a potential antigen was selected. This membrane was probed with T2 SCI plasma and a commercial anti-CRMP2 antibody. Some overlapping immunoreactivity between the patient plasma and anti-CRMP2 was expected if CRMP2 is a valid antigen.

The membrane was cut and scanned, wet with methanol, destained and blocked (in filtered PBST with 5%BSA, 1% milk) for 2 hours. The membrane was incubated overnight on a rocker at 4℃ in plasma T2 primary (1:500 in filtered PBST with 5% BSA, 1% milk). The membrane was then incubated in anti-CRMP2 raised in rabbit (1:1000, in filtered PBST with 5%BSA, 1% milk) for 8 hours. After washing in PBST the membrane was incubated with Alexa Fluor 488 (green) goat-anti-human IgG secondary (1:500) and Alexa Fluor 568 (red) goat-anti-rabbit IgG secondary (1:500) for 1 hour. This was followed by washing. To read the fluorescent signal, the membrane was placed protein side down on the Typhoon Trio scanner. The emission settings used for 50um scans were 5265P Fluor Cy Alexa Fluor, 488, PMT 375 and 610 BP30 green, 532, deep purple spyro ruby, PMT 375.

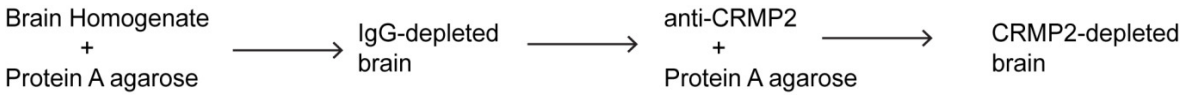
B. SCI plasma immunoreactivity to purified recombinant CRMP2

Western blots were performed as described in Chapter 2.7 to validate plasma immunoreactivity to CRMP2. SCI patient plasma samples known to be positive at T2 for the presence of autoantibodies found in the initial screening western blots were used to probe purified recombinant protein of human CRMP2. In this experiment 8% acrylamide gels were used in order to increase the separation of proteins between 37-75kDa. Purified recombinant CRMP2 (0.25µg/well) was used as the antigen that was probed with patient T2

plasma (1:500) or anti-CRMP2 (1:1000) followed by alkaline phosphatase secondary (1:50,000) and developed with West Pico.

C. CRMP2 immunodepletion of brain homogenate.

Figure 8. Flow chart of Immunodepletion of brain homogenate.



The goal of the immunodepletion was to have CRMP2-poor brain homogenate to use for western blot (Figure 8). Protein A agarose resin (Pierce) was used to remove the CRMP2 from brain tissue homogenate. The IgG from the brain tissue was depleted first to assure that donor brain tissue IgG was not contributing to immunoreactivity. 50µl of Protein A agarose resin was centrifuged, washed with 1ml PBST and centrifuged again to remove the 1ml wash. Brain supernatant (0.7µg/ul protein per microBCA) was added to 25µl resin beads. A second aliquot of 25µl resin beads was combined with 25µl anti-CRMP2 plus 261µl of 2%BSA in PBST to bring samples to an equivalent volume. Both were allowed to incubate overnight in the cold room on an agitator. After incubation these samples were centrifuged for 5 min at 500Xg, and each of the supernatants were divided in half. The CRMP2 beads were washed twice in 1ml PBST. Half of the IgG-depleted brain supernatant was combined with the anti-CRMP2 beads to deplete the CRMP2 from this sample. The supernatant and beads were allowed to incubate for 8 hours. Dot blots with anti-CRMP2 were used to confirm depletion of CRMP2 from the sample.

The IgG-depleted brain or the CRMP2-depleted brain supernatants (5µg protein/well) were run on an 8% acrylamide gel and proteins were transferred to an immobilon-P membrane. The membrane was probed with patient T2 plasma (1:500) or anti-CRMP2 (1:1000) followed by alkaline phosphatase secondary (1:50,000) and

developed with West Pico. These Western blots were run to determine whether the immunoreactivity produced by patient plasma that was shown to be immunoreactive to purified CRMP2 decreased when probing the CRMP2-depleted brain sample.

D. Capillary electrophoresis–immunoassay

After identifying patient plasma samples containing anti-CRMP2 immunoreactivity using western blots, the specificity of immunoreactivity was verified using a commercial anti-CRMP2 antibody and purified recombinant human CRMP2 protein with a capillary-electrophoresis immunoassay. All subject samples were then tested on this capillary-electrophoresis immunoassay. Purified recombinant protein CRMP2 (36ng/µl) was denatured with SDS-containing buffers and florescent standards and subsequently loaded into capillaries for separation by size. Ultraviolet light was used to immobilize the proteins to the capillary wall where they were probed with plasma primary (1:100) and anti-human HRP secondary 1:500 (Vector). Separation was run for 25 minutes at 375 volts, antibody diluent incubation time was 5 minutes, primary antibody incubation time was 30 minutes, secondary antibody incubation time was 30 minutes and detection used multi-image analysis of 5 exposures (1, 2, 5, 15 and 30 seconds). Chemiluminescence (produced by luminol and peroxide) was acquired by a charge-coupled device camera and the image was measured and peaks were detected by the system software (Compass for SW, ProteinSimple, V3.0.9). System parameters were set with a peak threshold of 10, width of 9 and a Gaussian fit for area calculation. Each assay included positive control capillaries of the commercial anti-CRMP2 antibody (1:1000) and an anti-rabbit secondary, a negative control of no primary antibody (antibody diluent only), and a known positive human plasma sample (identified through 1-D and 2-D gel testing). All time points for an individual subject were assayed by capillary electrophoresis -immunoassay on the same capillary array to

minimize between-assay variability for a subject. All immunoassays were performed on the same instrument.

The area under the curve (AUC) and the signal to noise ratio (S/N) were calculated with the assay software (Compass for SW, ProteinSimple, V3.0.9). Existence of a curve was confirmed by visual inspection. The measurements of AUC, in arbitrary units, were used for comparison between groups.

E. Determination of specificity of autoantibodies

To verify antigen-binding specificity, competition studies were performed on the diluted plasma (1:100) samples that were analyzed for reactivity to CRMP2 protein using the capillary electrophoresis-immunoassay. Plasma samples were incubated overnight at 4°C either with 360ng purified CRMP2 protein/10µl of plasma or a competing protein of a similar molecular weight that contained the same tag (Myc-DDK) used for purification (glial fibrillary acidic protein, GFAP or calreticulin, CALR) of an equimolar amount. Immunoreactive signals to CRMP2 obtained with the pre-absorbed samples (CRMP2, GFAP or CALR) were compared to immunoreactive samples from the plasma sample that was not pre-absorbed. Anti-CRMP2-specific immunoreactivity should be diminished if there is cross-reactivity with the CRMP2-pre-absorbed sample. Immunoreactivity from samples deemed anti-CRMP2-specific had to have a decrease in AUC. Immunoreactivity that had a similar decrease when pre-absorbed with GFAP and/or CALR was deemed non-specific. The presence of plasma anti-CRMP2 (CRMP2ab) was considered positive if 1) the AUC was above the level of background noise and 2) the AUC decreased greater than 30% when pre-absorbed with the CRMP2 protein, but not the pre-absorbed competing protein(s).

BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine) searches for homology between CRMP2 and GFAP or CALR were performed.

The BLAST search for CRMP2 and GFAP resulted in 3 matches, one of which had 6 sequential amino acids. The BLAST search comparing peptide sequences for CRMP2 and CALR identified two areas with overlapping peptide sequences, one had 8/20 the other 8/28 identical peptides, at most there were only 3 sequential amino acids.

F. Analysis of CRMP2ab levels by pain group

Patients were classified as positive or negative for neuropathic pain based on a clinical diagnosis of neuropathic pain as describe in Section 2.3. Acute SCI subjects were divided into those classified as positive for neuropathic pain within 6 months post-SCI versus those who did not have evidence of neuropathic pain by that time. Chronic SCI subjects were classified according to their pain status at the time of sampling. A comparison of the S-LANSS scores (as described in Chapter 2.3B) between pain groups was performed as a corroboration of the clinical diagnosis of neuropathic pain. The Mann-Whitney Rank-Sum Test was used to determine whether there was a difference in the levels of CRMP2ab at 16 ± 7 days in those with versus without neuropathic pain within 6 months of SCI.

3.3 Results

A. Fluorescent staining validates CRMP2 as a source of immunoreactivity

Probing the membrane with T2 plasma followed by anti-CRMP2 utilizing anti-human or anti-rabbit specific secondary antibody with fluorescent tags confirmed co-localization of SCI plasma and CRMP2 immunoreactivity (Figure 9). For further confirmation, calipers were used to make precise measurements of co-localized spot locations and spots from corresponding gels were cut and sent for repeat LC-MS/MS identification. The repeat LC-

MS/MS results confirmed the original CRMP2 findings. These findings implicate CRMP2 as a potential antigenic target of an autoimmune response after SCI.

Figure 9. CRMP2 Verification, Co-localization of Immunoreactivity.

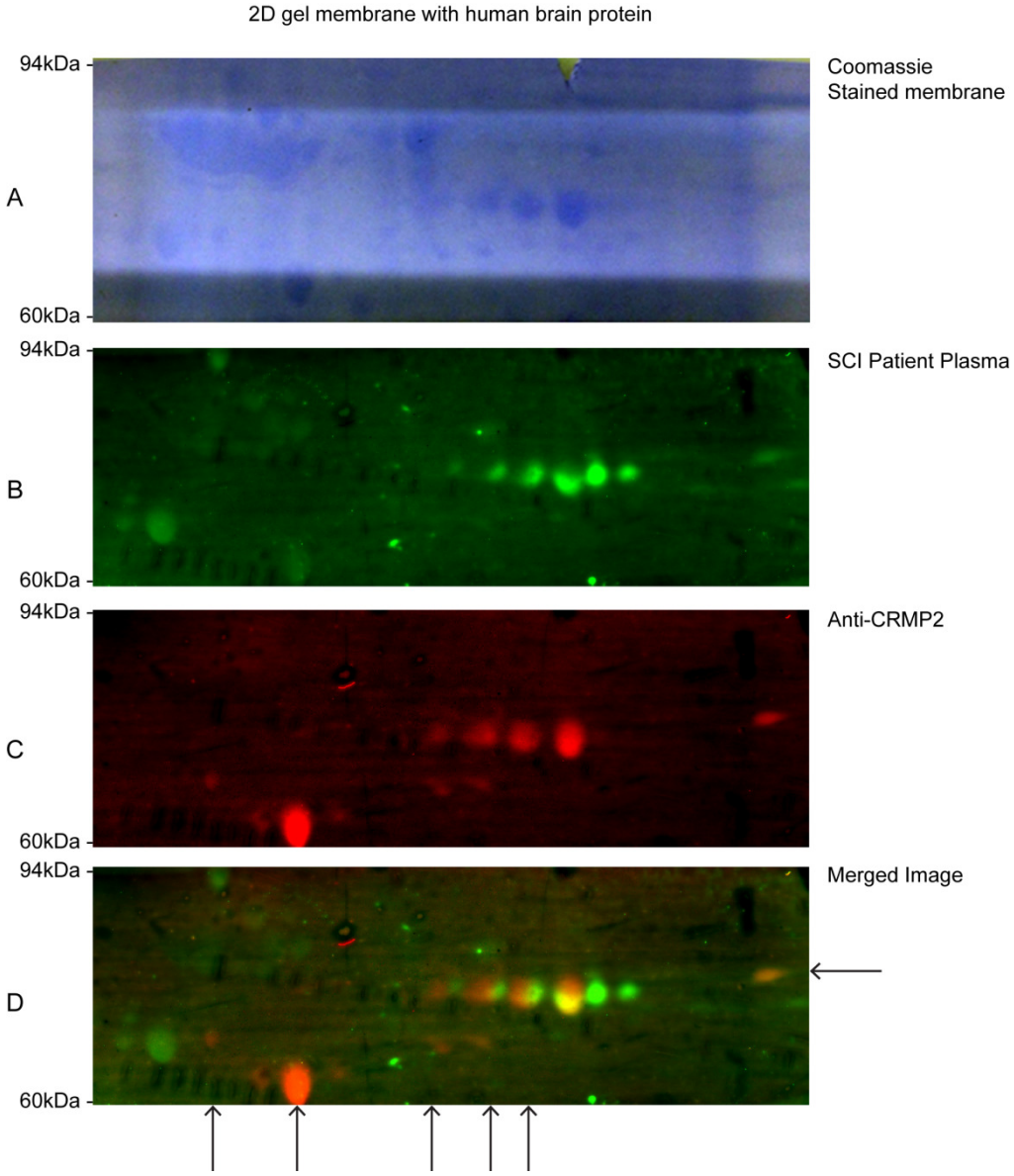


Figure 9. CRMP2 Verification, Co-localization of immunoreactivity.

Coomassie stained membrane after transfer from a 2-D gel containing CNS proteins (A). The membrane was probed with T2 SCI patient plasma known to be immunoreactive and Alexa Fluor 488 (green) goat-anti-human IgG secondary (B). The same membrane was also probed with anti-CRMP2 (rabbit) and Alexa Fluor 568 (red) goat-anti-rabbit IgG secondary (C). Arrows show co-localization of some of the fluorescent green and red spots (D).

B. Western blotting confirms T2 SCI plasma immunoreactivity to purified recombinant CRMP2.

Representative results are shown in Figure 10. The SCI plasma sample shown below is immunoreactive to CRMP2 at T2. The commercial anti-CRMP2 positive control developed a broad single band at the same molecular weight location. These results are consistent with the findings from the LC-MS/MS showing SCI patient plasma immunoreactivity to CRMP2.

Figure 10. Western blot showing patient plasma at T2 with banding pattern consistent with positive immunoreactivity to CRMP2 protein.

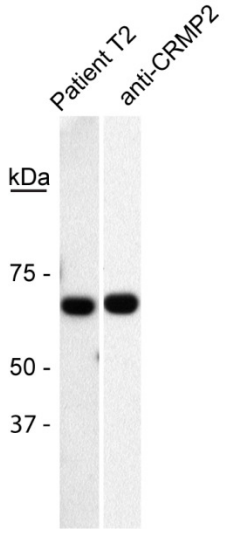


Figure 10. Western blot showing SCI patient plasma at T2 with banding pattern consistent with positive immunoreactivity to CRMP2 protein. Lane 1 shows Patient T2 plasma probed against purified recombinant CRMP2 protein. Lane 2 is a commercial anti-CRMP2 antibody. These results are consistent with the LC-MS/MS results that identified CRMP2 as a potential antigen.

C. Immunodepletion of brain tissue demonstrates a decrease in T2 plasma immunoreactivity at the molecular weight of CRMP2.

Immunoreactivity decreased in the CRMP2-depleted brain at the molecular weight level of CRMP2 bands. Representative results are shown in Figure 11.

Figure 11. Decreased Immunoreactivity of T2 Patient Plasma on CRMP2-depeleted brain sample.

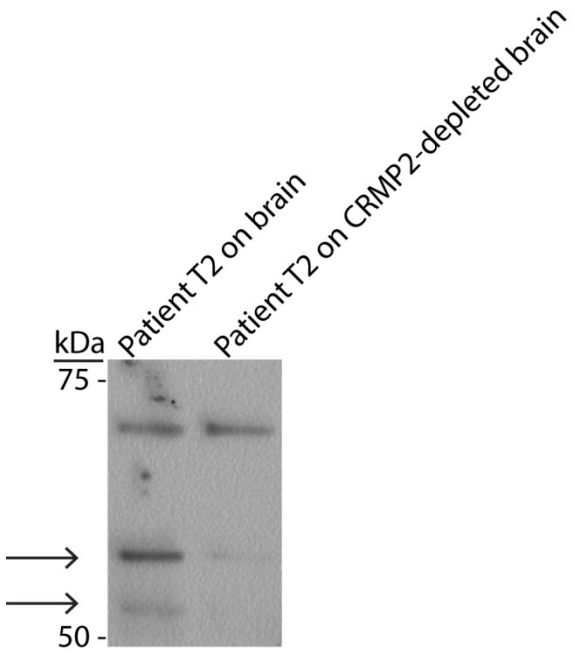


Figure 11. Decreased immunoreactivity of T2 patient plasma on CRMP2-depleted brain sample.

Protein A agarose resin beads were used to deplete brain homogenate of IgG and then further deplete the homogenate of CRMP2. Lane 1 shows immunoreactivity of a T2 SCI patient sample against IgG-depleted brain. On Lane 2 the same plasma was used to probe the brain sample that was depleted of CRMP2. The reduced immunoreactivity from patient plasma in Lane 2 suggests this patient plasma is immunopositive to CRMP2.

D. Capillary electrophoresis immunoassay is effective at measuring the SCI plasma for immunoreactivity to CRMP2

The previous experiments confirm that some SCI subjects produce immunoreactivity to the CRMP2 antigen that was identified through 2-D electrophoresis and LC-MS/MS. In order to estimate the percent of the acute SCI subjects who are positive for CRMP2ab purified recombinant CRMP2 protein was used as the sample on a capillary electrophoresis –immunoassay. Increasing dilutions of plasma and amounts of protein were tested to determine loading amounts. An illustration of the difference of immunoreactivity to CRMP2 from the same plasma sample at dilutions of 1:50 or 1:100 is shown in Figure 12. Plasma dilutions of 1:100, and CRMP2 protein at 36 ng/µl were used for these studies.

Figure 12. Example of assay for CRMP2 plasma dilutions and protein volume

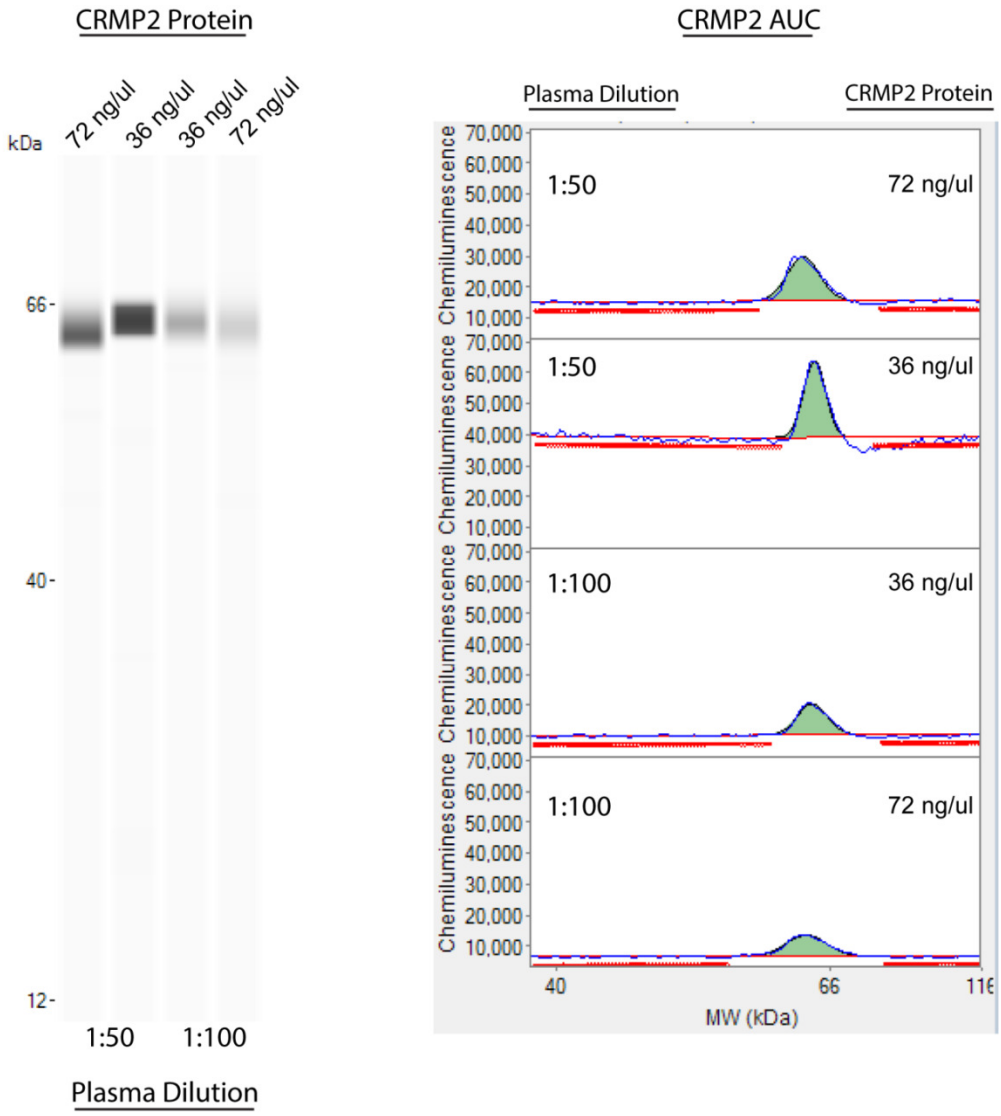


Figure 12. Preliminary capillary electrophoresis immunoassays were performed to determine the amount of protein and the dilution of plasma antibody to use for subsequent testing. The image on the left shows immunoreactivity from one plasma sample of one patient that was diluted either 1:50 or 1:100 used to probe CRMP2 protein 36 or 72 ng/µl. The image on the right shows the corresponding areas under the curve (AUC). Plasma dilutions of 1:100, and CRMP2 protein at 36 ng/µl was selected for subsequent studies.

The level of immunoreactivity produced by acute SCI patient plasma in response to CRMP2 protein was measured in serially collected plasma samples as the timing of production of autoantibodies was expected to have some variability in different patients. The 16 ± 7 day sample time point produced the peak immunoreactivity. Figure 13 shows an image of the immunoreactivity on the capillary electrophoresis immunoassay of samples at three time points from a SCI patient that was also immunopositive to CRMP2 on acrylamide gels.

Figure 13. Capillary electrophoresis immunoassay of patient plasma on CRMP2 protein.

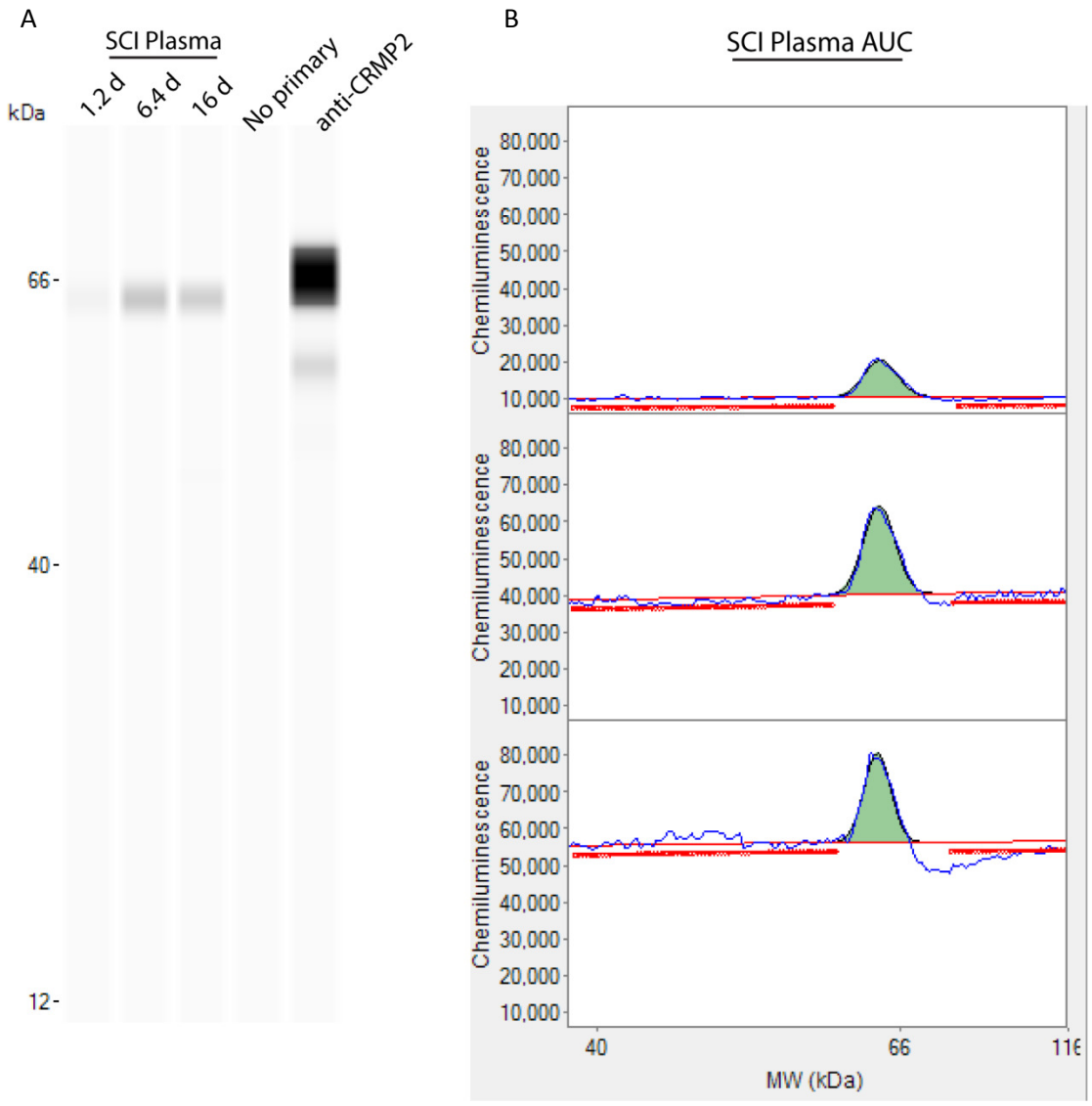


Figure 13. Capillary electrophoresis immunoassay of patient plasma on CRMP2 protein.

A) Immunoassay: Lanes 1-3 probed with patient plasma (1:100) at 3 time points on 36ng/µl of CRMP2. Lane 4 shows no immunoreactivity using no primary and anti-human HRP (1:500) secondary. The positive control commercial anti-CRMP2 (1:1000) is in Lane 5. B) Corresponding graph of immunoreactivity of SCI plasma. The green color identifies the area measured to calculate AUC of CRMP2ab.

E. Specificity of Immunoreactivity

E.1 Subjects used in antigen-binding specificity and subsequent studies

Acute, chronic and healthy volunteers were evaluated to test for the presence of CRMP2ab. A total of 138 subjects (20 healthy volunteers, 38 acute SCI and 80 chronic SCI) were consented and enrolled for their participation in this study. The demographics of these subjects are reported in Table 6. On average, the acute SCI patients were 1.2 days post-injury, whereas the chronic SCI patients were 15 years from injury. The primary cause of injury in the majority of SCI patients was motor vehicle accidents (MVA). The acute and chronic SCI groups included primarily cervical injuries and over half of each SCI group was ISNCSCI A or B impairment level (A: complete loss of function; or B: sensory, but no motor function was preserved below the level of the injury).

Acute SCI subjects were divided into those classified as positive for neuropathic pain within 6 months post-SCI versus those who did not have evidence of neuropathic pain by that time. Chronic SCI subjects were classified according to their pain status at the time of sampling. In both acute and chronic SCI groups, all subjects with an S-LANSS score of greater than 12 had a clinical diagnosis of neuropathic pain. The chronic subjects with neuropathic pain had a significantly higher S-LANSS score than those without neuropathic pain ($T=741$, median 9 vs. 0, $p<0.001$), but there was no difference on the McGill pain score. S-LANSS scores were available for 18 acute SCI subjects at 6 months, when combining these scores with the chronic subjects' scores, the combined S-LANSS scores were significantly different between those with a clinical diagnosis of neuropathic pain and those without neuropathic pain ($T=1091$, median 9 vs. 0, $p<0.001$) corroborating the clinical diagnoses of neuropathic pain (Figure 14). The affective descriptors (tiring-exhausting, sickening, fearful, and punishing-cruel) on the McGill pain score were significantly higher for the acute SCI patients with neuropathic pain compared to those without neuropathic pain (mean 3.5 ± 3.2 vs 0.2 ± 0.4, $p<0.04$). Of the 38 acute SCI subjects, 23 (60.5%) were

diagnosed as having neuropathic pain by the 6 month time point while 15 (39.5%) had no evidence of neuropathic pain. Key demographics of the acute SCI subjects by pain group are shown in Table 7.

Table 6. Demographics of subjects used for capillary-immunoassay studies

	ACUTE SCI SUBJECTS (N=38)	CHRONIC SCI SUBJECTS (N=80)	HEALTHY VOLUNTEERS (N=20)
GENDER, COUNT (PERCENT)			
MALE	34 (89)	59 (74)	16 (80)
FEMALE	4 (11)	21 (26)	4 (20)
AGE, AVERAGE YRS(SD)	43.5 (17.7)	44.1 (14.0)	35 (13.2)
AGE, RANGE YEARS	18-82	19-76	20-62
RACE, COUNT (PERCENT)			
WHITE	32 (84)	66 (82)	11 (55)
ASIAN	0 (0)	3 (4)	6 (30)
BLACK	5 (13)	11 (14)	3 (15)
OTHER	1 (3)	0 (0)	0 (0)
ETHNICITY			
HISPANIC	6 (16)	19 (24)	0 (0)
NOT HISPANIC	32 (84)	61 (76)	20 (100)
MECHANISM OF INJURY, COUNT (PERCENT)			N/A
MVA	23 (60)	47 (59)	
FALL	10 (26)	19 (24)	
SPORTS-RELATED	4 (11)	10 (13)	
ASSAULT	1 (3)	4 (5)	
TIME POST-SCI AT ENROLLMENT			N/A
AVERAGE (SD)	1.2 DAYS	15.3 (12.3) YEARS	
RANGE	0.1-2.7 DAYS	1- 41 YEARS	
LEVEL OF INJURY			
CERVICAL	31 (82)	47 (60)	
THORACIC	6 (16)	22 (28)	
LUMBAR	1 (2)	4 (5)	
UNKNOWN		7 (9)	
ISNCSCI Impairment Scale			
A	23 (60)	28 (35)	
B	3 (8)	21 (26)	
C	8 (21)	11 (14)	
D	4 (11)	12 (15)	
N/A		8 (10)	

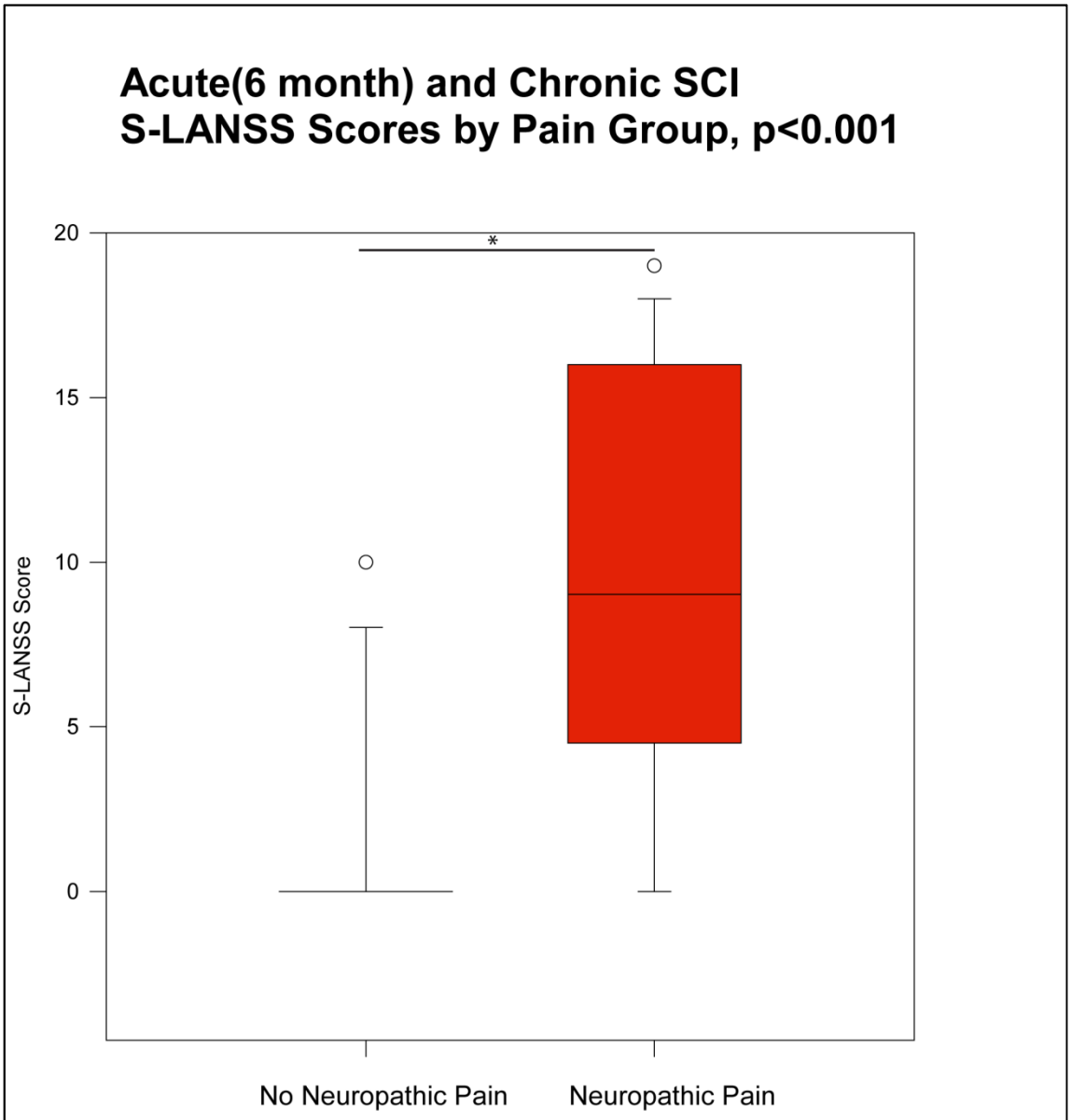


Figure 14. Box plot of S-LANSS Pain scores by pain group for Acute SCI and Chronic SCI patients. Acute SCI patients were classified as having neuropathic pain within 6 months of injury and chronic subjects were classified based on their pain state at the time of the visit (on average 15 years post-injury). Neuropathic pain classification was based on the clinical diagnosis of neuropathic pain and the S-LANSS score reported by patients supports the classification.[121] (T=1091, median 9 vs. 0, $p<0.001$)

Table 7. Demographics of acute SCI by pain group

	Neuropathic Pain (n=23)	**No Neuropathic Pain (n=15)**	**P***
Age (years, median)	36	47	0.69
BMI (kg/m2, median)	26.5	27.8	0.94
Male gender	19	15	0.14
Complete (ISNCSCI A)	14	9	1.0
Cervical Level Injury	15	14	0.06

*Mann-Whitney rank sum test for variables not normally distributed. Fisher's exact test was used for variables with small expected cell number. No significant results were found for any variables.

E.2 Specificity of Immunoreactivity to CRMP2

Specificity of immunoreactivity to CRMP2 was determined by pre-absorption of the plasma with purified CRMP2 or a non-specific protein (i.e. GFAP or CALR) which contained the same tag (Myc-DDK) used for purification. Only samples with immunoreactivity that was blocked by the pre-incubation with CRMP2, but not GFAP or CALR, were considered positive for CRMP2ab (Figure 15). Samples that demonstrated reduced immunoreactivity to both blocking (CRMP2) and competing (GFAP or CALR) proteins were considered nonspecific and therefore not counted as CRMP2ab positive (Figure 16). Only those samples that 1) had immunoreactivity above baseline, 2) decreased > 30% when pre-blocked with CRMP2 and 3) did not decrease when pre-absorbed with GFAP or CALR were deemed as having specific immunoreactivity to CRMP2 and were classified as CRMP2ab positive.

Figure 15. SCI patient plasma immunoreactivity specific for CRMP2.

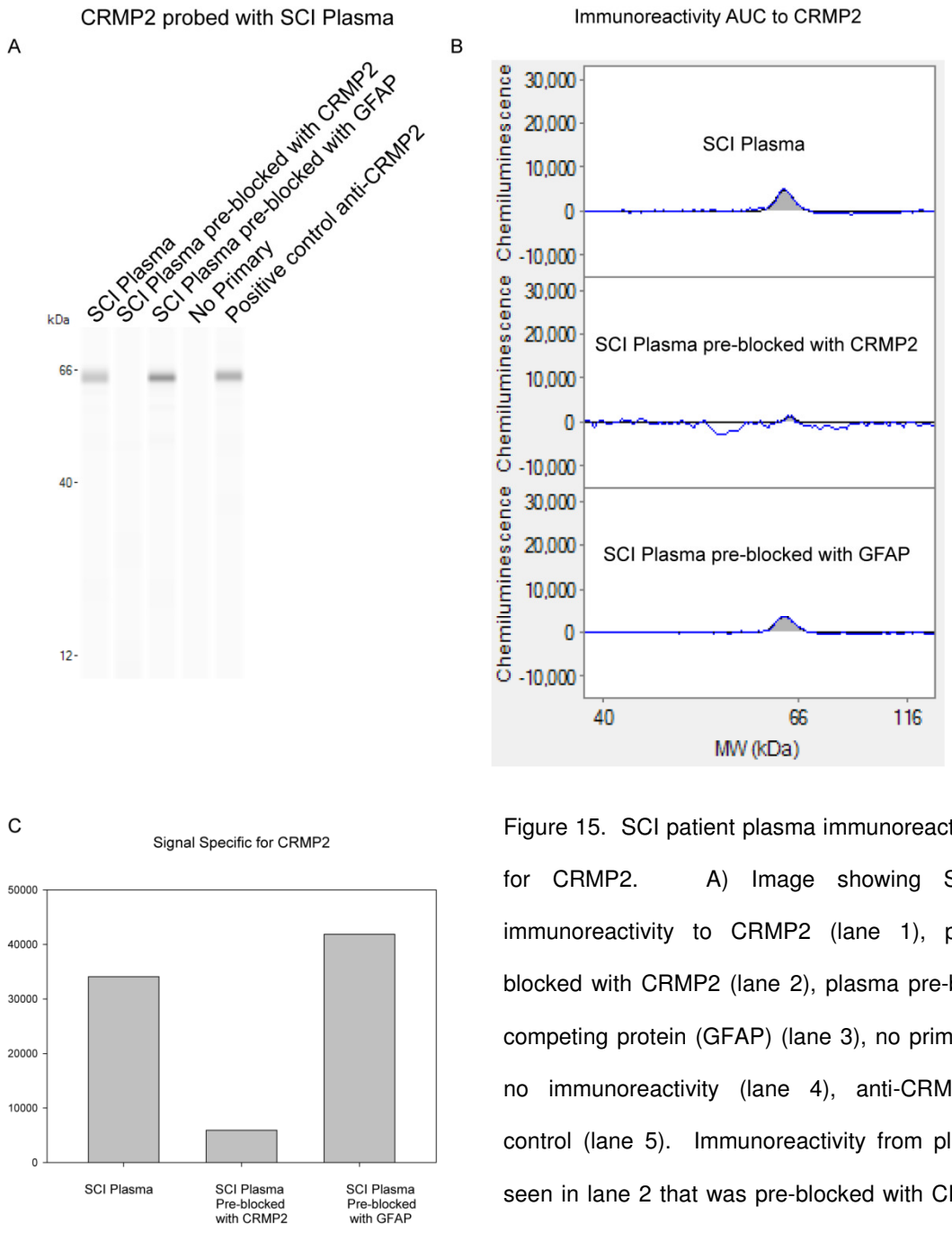


Figure 15. SCI patient plasma immunoreactivity specific for CRMP2. A) Image showing SCI plasma immunoreactivity to CRMP2 (lane 1), plasma pre-blocked with CRMP2 (lane 2), plasma pre-blocked with competing protein (GFAP) (lane 3), no primary showing no immunoreactivity (lane 4), anti-CRMP2 positive control (lane 5). Immunoreactivity from plasma is not seen in lane 2 that was pre-blocked with CRMP2, but it is present in the plasma that was pre-blocked with GFAP showing the plasma antibody in this sample is specific for CRMP2. B) AUC of plasma, pre-blocked plasma and competed plasma. C) Graphic representation of immunoreactivity change with blocking.

Figure 16. CRMP2 Blocking and Competing Studies – SCI patient non-specific for CRMP2.

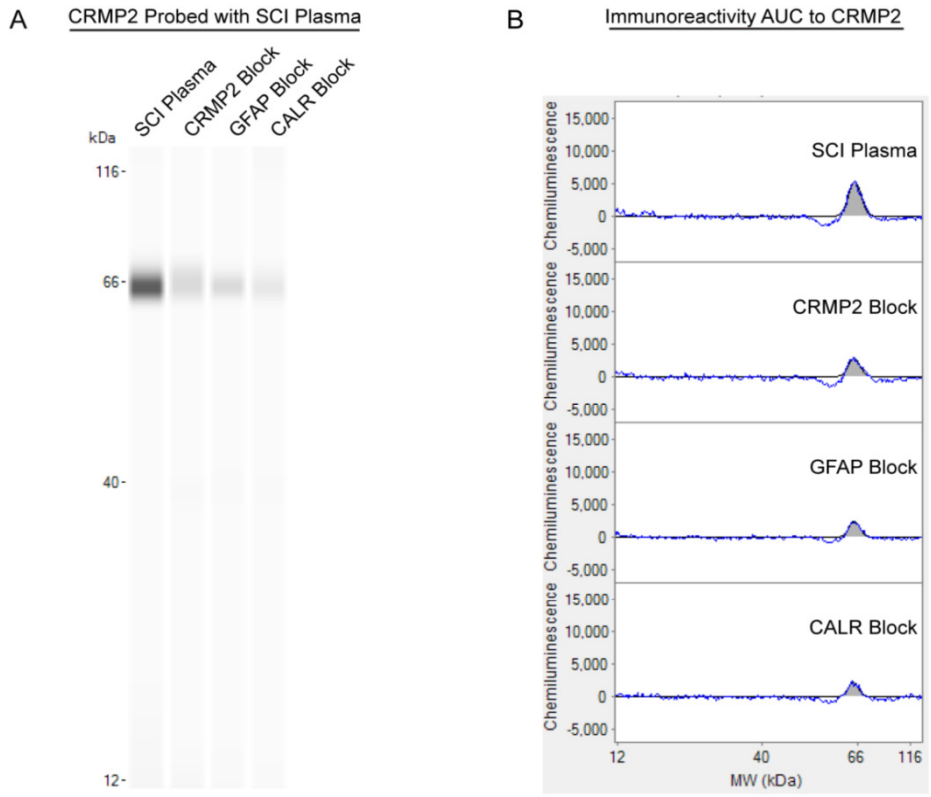


Figure 16. CRMP2 Blocking and Competing Studies – SCI patient non-specific for CRMP2. SCI plasma was incubated overnight in standard block (1:100) or equimolar amounts of CRMP2, GFAP or CALR protein. These samples were then used to probe CRMP2 protein. A) Lane 1 shows plasma immunoreactivity to CRMP2, Lane 2 the plasma was pre-blocked with CRMP2 and immunoreactivity was reduced as expected. Lane 3 the plasma was pre-blocked with GFAP and Lane 4 the plasma was pre-blocked with CALR. Pre-blocking with these competing proteins also reduced immunoreactivity indicating this response is non-specific. B) AUC of the immunoreactivity, C) Graphic representation of immunoreactivity change with blocking.

The Grubbs' test was performed to determine whether any samples in either group were outliers. One healthy volunteer was found to be a significnat outlier ($Z=2.71$, $p<0.05$) and was removed from analysis. Results indicated 8/35 (23%) SCI subjects and 1/19 healthy controls (5%) were CRMP2ab positive. However, the difference in the median levels of CRMP2ab was not significantly different between SCI subjects at the 16 ± 7 peak time point and healthy controls ($T=461.0$, $p=0.09$). CRMP2ab was detected in chronic SCI subjects, however because the peak acute timepoint did not reach significance the antigen verification studies were halted.

F. No statistically significant difference in the levels of CRMP2ab at 16 ± 7 days in acute SCI patients who developed neuropathic pain versus those who did not.

The results of the CRMP2 antigen verification and competition studies above identified 8 of 35 (23%) subjects had immunoreactivity that was specific for CRMP2 protein at 16 ± 7 days after SCI. (Three subjects with immunoreactivity did not block with CRMP2 and were omitted; the 6 day sample was used for the one subject who did not have a sample at 16 ± 7 days.) There was no difference in median CRMP2ab levels of those with and without neuropathic pain ($T = 231.000$, $p = 0.08$) (Figure 17). Sample size calculations based on data from theses acute SCI subjects indicate that a total of 64 subjects would be needed to detect a significant difference at an 80% power with an alpha of 0.05.

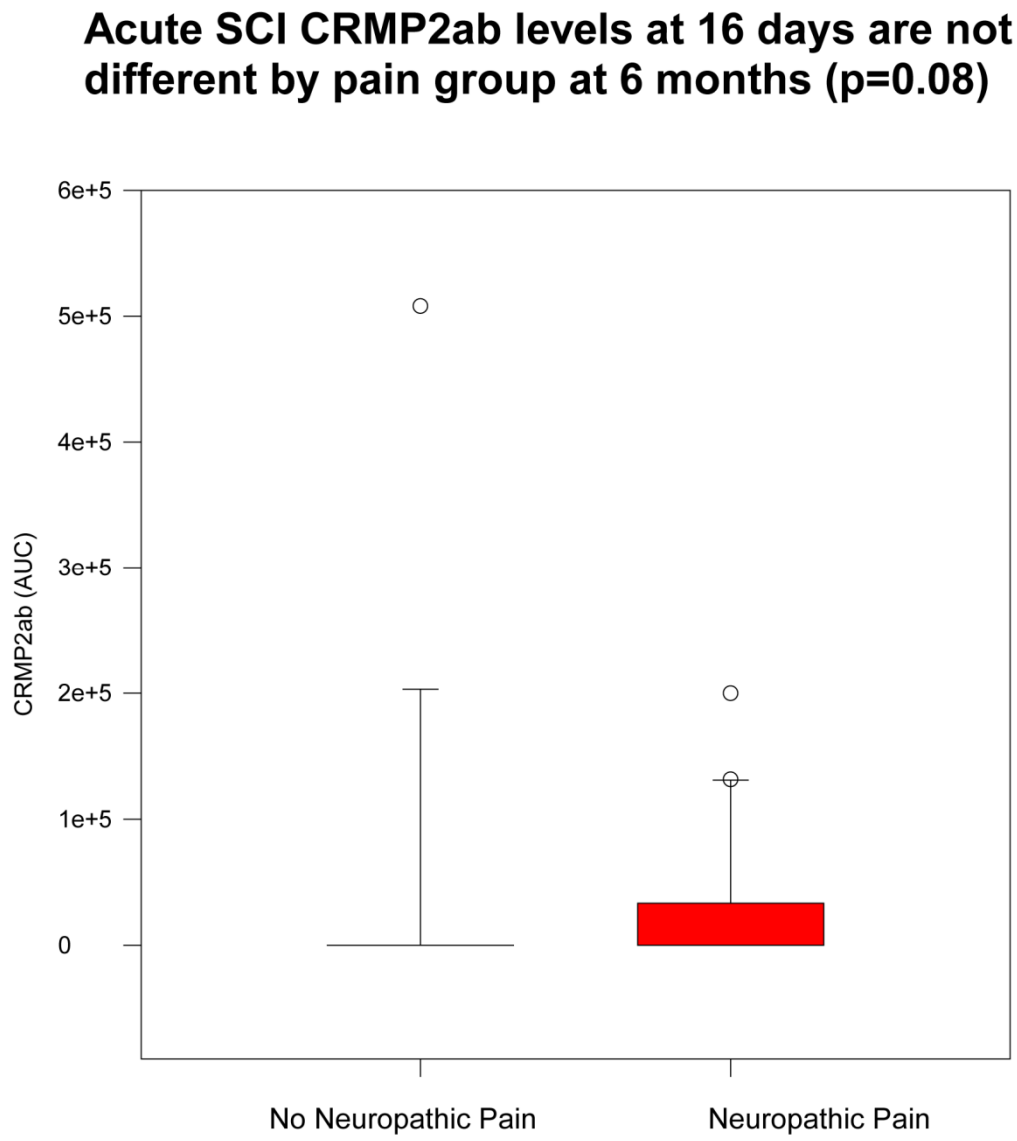


Figure 17. CRMP2ab levels at 16 ± 7 days after SCI were not statistically different between those who developed neuropathic pain (n=20) compared to those who did not develop neuropathic pain (n=15) within 6 months after SCI (T=231.000, p=0.08).

3.4. Discussion

A. Key findings

Eight of thirty-five (23%) subjects were identified as CRMP2ab positive after SCI. There was no difference in median CRMP2ab levels of those with and without neuropathic pain ($T = 231.000$, $p = 0.08$). Although this did not reach significance, it could be due to limited number of positive patients. Sample size calculations based on data from theses acute SCI subjects indicate that a total of 64 subjects would be needed to detect a significant difference at an 80% power with an alpha of 0.05.

B. Spots observed

Of the 22 spots sent for evaluation, 11 were positive for CRMP2. Of the spots matched to CRMP2, the molecular weight and species were correct. Figure 8 above, reveals spots that are likely different isoforms and post-translational modifications of CRMP2. There are multiple isoforms of CRMP2 including 75kDa CRMP2A and 62-66kDa CRMP2B variants that are commonly observed in adult CNS tissue.[139] One of the spots showed 59% coverage and the coverage included peptides in the N (glutamine 8, Q8) and C terminals (lysine 465, K465) supporting that it was identifying CRMP2, not homologous parts of other isoforms. CRMP2 has been identified as a potential antigen causing human plasma immunoreactivity previously on 2-D gels as a 62kDa protein with two phosphorylated isoforms at 64kDa and 66kDa.[155] Additionally, 55kDa CRMP2 breakdown products have been previously identified.[156] Spots that are more negatively charged (more phosphorylated) migrate to the left/acidic side of the 2-D gel.[141] Cyclin-dependent kinase 5 (CDK5) phosphorylates CRMP2.[157] In a chick SCI crush injury study CDK5 was inhibited with roscovitine in explanted cells. Using βIII-tubulin staining neurite sprouting and cell migration was detected, and in some cells there was an increase in phosphorylation at S522 mediated by CDK5. Two known sites of phosphorylation of CRMP2 are at threonine-

514 (70 and 78kDa) and serine-522 (62 and 70 kDa); phosphorylation at both residues was shown at 70 and 78kDa in chick spinal cord.[141]

C. Mechanism(s) by how CRMP2 autoantibody may contribute to neuropathic pain

CRMP2 autoantibody may contribute to neuropathic pain by fostering the sprouting of pain fibers. Aberrant sprouting of afferent fibers occurs after SCI. Calcitonin gene related peptide (CGFP) in the dorsal horn is a nociceptive neurotransmitter.[150] After SCI neurite spouting measured by increased density of growth associated protein-43 (GAP-43) co-localized with α-CGRP indicating nociceptive primary afferent sprouting in laminae where these primary afferents are not normally found.[150] In human postmortem subjects immunohistochemistry and immunocytochemistry for CGRP were performed on thoracic spinal cord tissue comparing 4 chronic SCI spinal cords below the level of the lesion and 5 intact control spinal cords. Confirming rodent studies, CGRP-containing fibers were found in both groups in lamina I and II, but in the human SCI tissue, fibers were denser and extended into deeper laminas III-V, VII and X.[158] Previous experimental rat studies demonstrated CGRP nociceptive sprouting was associated with hyperalgesia and allodynia.[151]

Chapter 4. Human SCI elicits an autoantibody response to GFAP

4.1. Introduction

A. Astrocytes

Astrocytes are the most abundant of central nervous system (CNS) glial cells.[159] Astrocytes provide structural support for neurons and facilitate the uptake of neural transmitters and maintenance of the blood-spinal cord barrier (BSCB). Astrocytes also control the migration of immune-mediating leukocytes into and out of the injured spinal cord.[160] Astrocytes can exert chemical signals that recruit leukocytes into the spinal cord parenchyma (pro-inflammatory) or restrict their entry (anti-inflammatory effect).[160] For instance, vascular endothelial growth factor A (VEGFa), derived from astrocytes, induces permeability of the BSCB and entry of leukocytes.[161]

Astrocytes are intricately involved in the BSCB providing chemical support for endothelial cells and maintaining the ion balance.[160] After trauma to the spinal cord, astrocytes are activated by cytokines and growth factors (e.g., IL-1, IL-6, TGFα, TGF-1, bFGF) becoming reactive.[160] Two types of reactive astrocytes have been identified, A1 and A2. A1 astrocytes are generally considered neurotoxic generating a pathological response whereas A2 astrocytes stimulate neuronal survival and healing of injured tissue.[162] Reactive astrocytes surround the injured area creating a glial scar.

The glial scar is thought to be protective by sequestering the damaged area and promoting cleanup of myelin and cellular debris, but the glial scar prevents axon regeneration across the scar, inhibiting recovery.[163, 164] After SCI astrocytes hypertrophy and form a mesh-like barrier around the lesion core.[165] Confining the inflammatory cells within the glial scar protects intact cells from macrophage-induced damage.[166] The reactive astrocytes forming the glial scar recruit inflammatory mediators

(chemokines, cytokines, growth factors, prostaglandin, nitric oxide) and perform a barrier function, restricting the spread of inflammation to uninjured tissue (Figure 18).[160] Consistent with this, reactive astrocytes that were experimentally ablated near the injury site by the antiviral ganciclovir in transgenic herpes simplex virus mouse stab and crush injury models demonstrated markedly worse tissue damage, demyelination, increased leukocyte infiltration and poorer motor function compared to control injured mice.[167, 168] GFAP is essential for astrocytes' glial scar formation.[169]

Astrocytes are involved with autoimmune pathology. For example, the disruption of astrocyte functions by binding of complement and autoantibodies that bind to aquaporin 4 (AQP4) on astrocyte membranes is causal of neuromyelitis optica (NMO), a demyelinating disease of the eye which leads to loss of vision.[160] The AQP4-autoantibodies result in complement-mediated astrocyte damage, blood-brain barrier breakdown, increased inflammation and enlarged tissue damage.[160] Furthermore, in NMO astrocyte-associated damage of the optic nerve and spinal cord can cause pain.[170]

Gliopathy, defined as "the dysfunctional and maladaptive response of glial cells to neural injury" is proposed to be the result of the sudden increase in extracellular glutamate concentration resulting in excitotoxicity, sensitization of neurons and glia and includes increase of pro-inflammatory cytokines and immune cell infiltration.[171] Gliopathy results in ongoing inflammation and neuropathic pain.[171]

Figure 18. Model of spinal cord injury showing reactive astrocytes creating a glial scar.

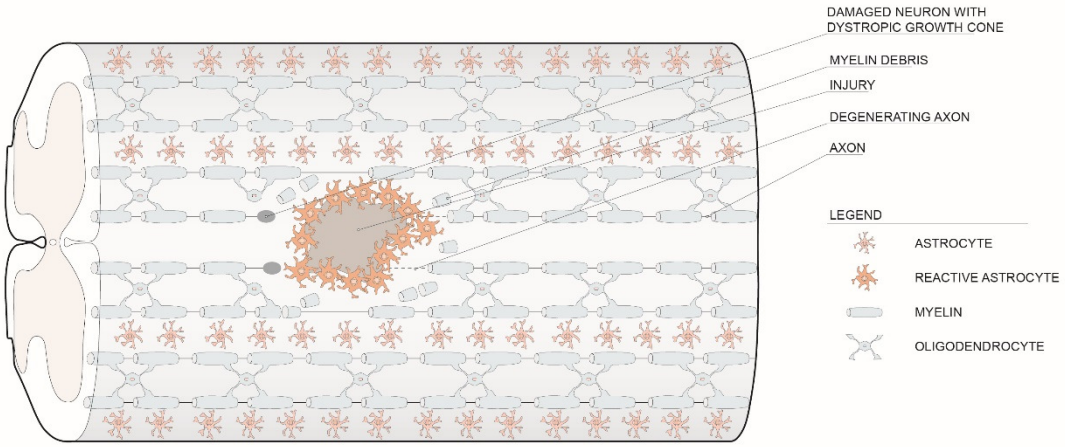


Figure 18. Model of spinal cord injury showing reactive astrocytes creating a glial scar.

A spinal cord injury causes damage to neurons resulting in dystrophic growth cones, degenerating axons and cellular debris.

After trauma to the spinal cord, astrocytes are activated by cytokines and growth factors becoming reactive astrocytes in response to the trauma. Reactive astrocytes surround the area of injury creating a glial scar. The glial scar is protective by sequestering the damaged area and promoting clean up, but also inhibits axons ability to regenerate across its inhospitable territory.[166] The model shows activated astrocytes surrounding the injury site.

B. The Role of GFAP

GFAP knock-out mouse models have shown reduced glial scar formation in GFAP knock-out mice.[169, 172] This weakened glial scar may contribute to a persistently permeable BSCB, or it may promote axonal growth across the lesion.[172]

DNA microarray analysis on rat spinal cord after SCI found upregulated genes associated with inflammation and astrocyte activation in rats with SCI and chronic pain compared to SCI rats without chronic pain.[25] GFAP mRNA and protein expression were elevated in SCI and further increased (more than 2-fold) in SCI rats with chronic pain. The increase in GFAP expression started as early as 4 hours after injury and persisted for 9 months in chronic pain SCI rats. Activation of astrocytes and the accompanying pro-inflammatory state is associated with chronic pain. The authors propose that persistent astrocyte hypertrophy leads to (and/or results from) the breakdown of the BSCB and entry of inflammatory cells into the injured cord, ultimately contributing to the onset and maintenance of neuropathic pain.[25]

C. GFAP is released after CNS injury

GFAP protein is known to be released after SCI and TBI and is proposed to be a biomarker for CNS injury.[173-176] The release of GFAP, a CNS antigen, after SCI could disrupt the state of immune tolerance and result in autoantibody production. The process of the development of autoantibodies after SCI (a “sterile injury”) is proposed to be similar to immune response to pathogen associated molecular patterns (PAMPs) in bacterial exposure; however, after a sterile injury development of autoantibodies is in response to damage associated molecular patterns (DAMPs).[177, 178] Circulating B cells are recruited by the dying cells, B cells can bind the released self-antigen (GFAP) and the B cell can be activated by a T cell that is specific for a self-peptide. These B cells differentiate

into plasma cells secreting autoantibody, the autoantibody further stimulates the inflammatory response inducing more cell damage and activating more autoreactive B cells, producing more autoantibody until the damaged cells are cleared.[179] Deficiencies in the clearance of apoptotic and dying cells after SCI may contribute to loss of B cell tolerance and result in autoantibody production.[180]

After injury, inflammation is induced in recognition of damage associated molecular patterns (DAMPs) on the released CNS antigen(s) (e.g., GFAP and CRMP2) that are detected by pattern-recognition receptors (PRRs) (similar to the mechanism used by pathogens and PAMPs).[178] Classes of PRRs include Toll-like receptors, NOD-like receptors, anti-viral RIG-I-like receptors, C-type-lectin receptors with their carbohydrate-binding domain and absence in melanoma 2-like receptors (the latter PRR having involvement in immune responses to bacteria and DNA viruses); PRRs mediate the DAMP-associated inflammatory response.[180] After tissue damage, PRRs activate signaling pathways mitogen-activated protein kinase (MAPK), nuclear factor-kB (NF-kB), and type 1 interferon (IFN-1) which increase pro-inflammatory cytokines (e.g., IL-1, TNF) and chemokines.[181] IL-1α and IL-33 are released in their precursor, biologically active form from cells during necrosis leading to the recruitment of neutrophils and alarmins which alert immune cells.[181] Uric acid is an example of a pro-inflammatory DAMP that contributes to inflammation.[182] Post-trauma necrotic cell death resulting in the loss of cell membrane integrity results in the release of multiple pro-inflammatory endogenous DAMPs including high-mobility group box 1 (HMBG1), heat shock proteins, S100 proteins, heparin sulfate, DNA, RNA and others.[181, 183]

Heat shock proteins, S100, uric acid, HMGb1 and heparin sulfate activate Toll-like receptors expressed on the membranes of dendritic cells and lymphocytes.[181] Dendritic cells are activated by necrotic cells releasing heat shock protein 70, IL-1α, HMGB-1 which induce Toll-like receptor-IL-1 signaling.[184] Dendritic cells then present the auto-antigen

resulting in the potential for formation of autoantibodies. The development of autoantibodies has been demonstrated in a sterile injury mouse model of myocardial infarction which results in ischemia and tissue necrosis causing release of intracellular contents that cause sterile inflammation.[185] The mouse myocardial infarction model proved that dendritic cells presented the self-antigen to autoreactive CD4+ T cells which assumed an autoreactive T-helper cell phenotype, Th1/Th17, promoting autoantibody production.[184]

DAMPs can bind to an antibody and activate complement through antibody binding to lipids on the necrotic cell surface, or to released antigen.[177] Complement is also activated by proteases released from damaged tissue.[186] The complement cascade results in the activation of inflammatory anaphylatoxins C3a and C5a.[187, 188] Mice deficient in C3 have a reduced immune response compared to wild type mice.[177] However, even in the absence of complement, in the context of cell death B cells and antibodies contribute to inflammation, possibly through uric acid promoting inflammation or through antibody binding Fc receptors on leukocytes.[177] PAR2 receptor expressed on leukocytes can induce inflammation and it can be activated by proteases released from necrotic cells; consistent with this, PAR2 deficient mice had reduced inflammatory response compared to wild type mice in a cell death-induced model.[177] The PRRs that activate the Toll-like receptor pathway (e.g.,TLR7 with IFN-1) cause the activation of B cells and production of autoantibodies.[189, 190] GFAP that is released after injury could similarly cause the activation of B cells and the production of autoantibody.

Increased GFAP expression after CNS injury accompanies astrocyte hypertrophy and activation. Activation of astrocytes leads to the secretion of inflammatory mediators (IL-1β, IL-6 and TNF-α) and expression of inducible nitric oxide synthase which propagates the inflammatory state and may potentiate SCI-induced chronic pain.[25]

The development of an autoantibody that binds to GFAP may contribute to prolonged BSCB breakdown, continued passage of inflammatory mediators across the BSCB and potentiate pain. The hypothesis is that autoantibodies to GFAP will be present after SCI and will be associated with neuropathic pain. The following experiments were performed to validate the potential of GFAP to be an antigen to which some SCI patient plasma is immunopositive and assess the relationship to neuropathic pain.

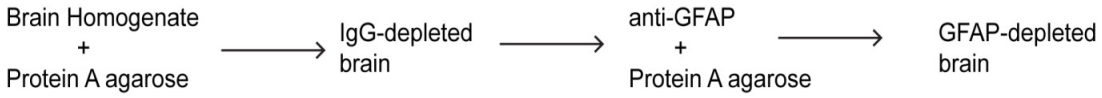
4.2. Methods

A. Spot excision and LC-MS/MS

Immunoreactive spots from 2-D gels were identified and excised from Coomassie stained gels as described in Chapter 2.13B Proteins were subjected to LC-MS/MS and identified as described in Chapter 2.13C.

B. Validation of GFAP antigen, Immunodepletion and western blots

Figure 19. Flow chart of GFAP immunodepletion of brain homogenate



Immunodepletion was performed to prepare IgG-poor and GFAP-poor brain homogenate as described in Chapter 3.2 C; 25µl anti-GFAP was used in these experiments to create the beads to deplete GFAP from brain homogenate (Figure 19).

To further validate that the T2 patient samples produced immunoreactivity to GFAP, western blots were performed as described in Chapter 2.7. These western blots were from 8% acrylamide gels containing purified recombinant GFAP (5µg/well) (OriGene).

C. Capillary electrophoresis immunoassay and antigen verification

Western analysis and verification of antigen specificity was carried out as described in Chapter 3.2 D and E. The capillary electrophoresis immunoassay parameters were the same except GFAP protein (160ng/µl) and plasma dilutions of 1:50 were used and a custom anti-GFAP antibody (1:20,000) was the positive control. Those plasma samples that were immunopositive to GFAP were considered as having autoantibodies reactive to GFAP (GFAPab) if they met the criteria of 1) having immunoreactivity above baseline, 2) decrease by >30% when pre-blocked with GFAP protein, and 3) immunoreactivity did not decrease when blocked with a competing protein. Subject samples used in these studies are described in Chapter 3.2 E.1 and Tables 6 and 7.

D. Receiver Operator Characteristic (ROC) curve analysis

A ROC curve was generated to determine the sensitivity (true positive rate) and specificity (1- specificity is the false positive rate) of GFAPab at predicting neuropathic pain within 6 months of SCI at different thresholds. The area under the curve (AUC) generated from the ROC curve provides an index of how useful GFAPab is at predicting neuropathic pain.[191] An AUC of 0.5 has no discriminatory ability; generally, an AUC > 80 is good, 70 to 80 is fair, and < 70 is considered a poor predictor.[191]

To estimate the ng/mL of GFAPab present in human plasma a standard curve was created with the custom anti-GFAP antibody that had a known concentration. The GFAP sample (160ng/mL) was probed with the custom anti-GFAP antibody (1mg/mL) at increasing dilutions (1:25,000 to 1:3200,000). A standard curve of the anti-GFAP AUC immunoreactivity relative to anti-GFAP ng/mL was generated (Figure 20). This curve was used to estimate the concentration of GFAPab (ng/mL) in plasma from the AUC measurement that was predictive of future development of neuropathic pain. The

concentration of GFAPab that provided the highest sensitivity and specificity was selected as the threshold value.

Figure 20 Extrapolation of GFAPab threshold in ng/mL based on GFAPab AUC

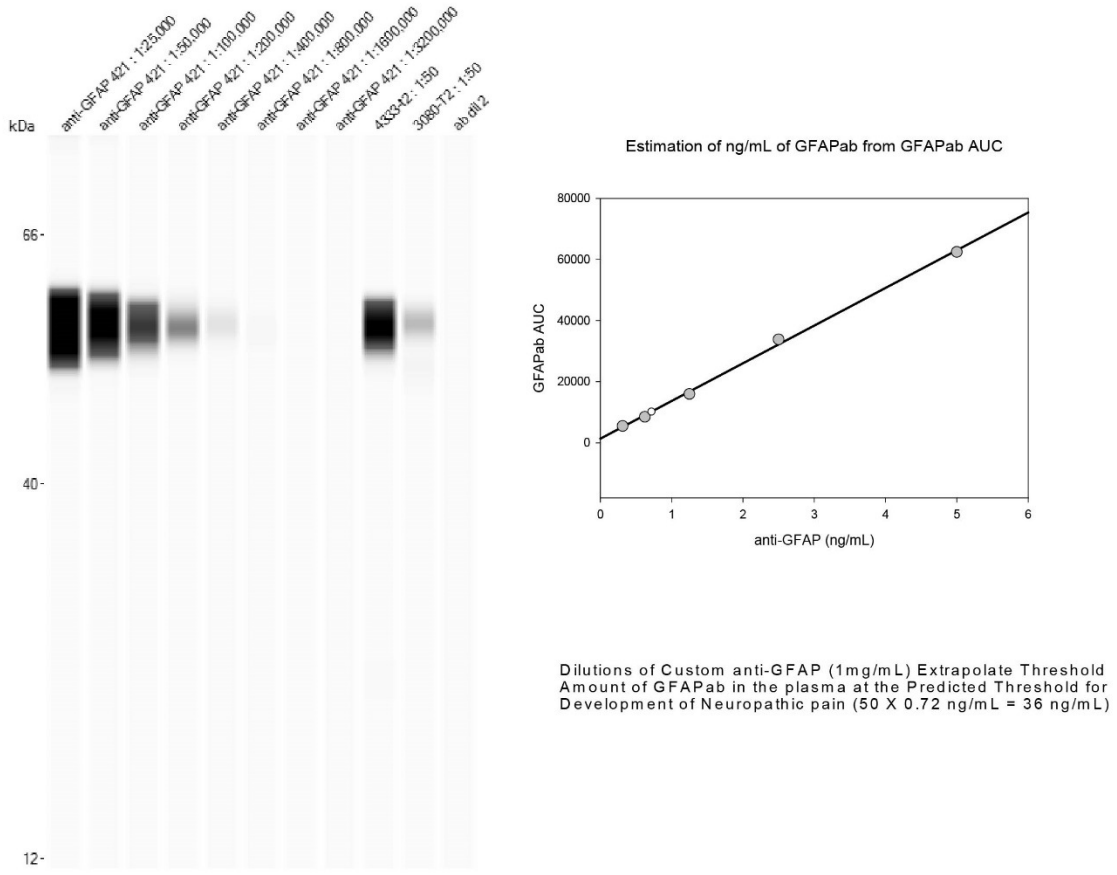


Figure 20. Extrapolation of GFAPab threshold in ng/mL. The GFAP protein sample (160ng/mL) was probed with a custom anti-GFAP antibody (concentration of 1mg/mL) at increasing dilutions (1:25,000 to 1:3200,000) (lanes 1-8). Two SCI plasma samples (1:50 (lanes 9-10) and no primary (lane 11) were included as controls. A standard curve of the anti-GFAP AUC immunoreactivity relative to anti-GFAP ng/mL was generated (shown above right). The white circle indicates the threshold level of GFAPab in patient plasma at 16±7 days that would be predictive of development of neuropathic pain within 6 months of injury.

E. Measuring the odds of developing of neuropathic pain when GFAPab and/or CRMP2ab are present at 16 ± 7 days.

The presence of the two autoantibodies on the the development of neuropathic pain was analyzed using a chi-square test and an odds ratio was calculated. Multiple logistic regression was performed while controlling for age, gender, body mass index, complete injury, and cervical level to evaluate the probability of the GFAPab and/or CRMP2ab predicting neuropathic pain. Statistical analysis was performed as described in Chapter 2.11.

4.3. Results

A. Spots excised identified as GFAP.

Seven of the spots sent for LC-MS/MS analysis were positive for GFAP (Chapter 2.13B). The species was correct. Results of peptide matches showed good coverage. For example, on one spot the sequence coverage was 66.8%. For that spot, the first matched peptide started with serine 8 (S8) in the N terminal region and the last matched peptide ended with Lysine 399 (K399) which is in the C terminal region; additionally there were multiple peptides matched in the rod domain.[192] Consistent with GFAP, these spots ranged in molecular weight from 38 to 50kDa and *pI* of between pH 5 to 6. The original 2-D gel membrane that was used to identify spots for LC-MS/MS analysis was stripped and re-probed with a custom anti-GFAP antibody. There was overlapping reactivity between the index SCI patient immunoreactivity and the immunoreactivity produced by the custom anti-GFAP antibody. This suggested that the patient IgG immunoreactivity and the anti-GFAP antibody had some commonality.

B. Validation of immunoreactivity to GFAP.

The brain homogenate and GFAP-depleted homogenate were probed on a western blot with T2 patient plasma known to be positive for immunoreactivity at 38-50kDa. Brain homogenate sample depleted of GFAP had less immunoreactivity between 38-50kDa than the non-GFAP depleted sample. Western blots probed with SCI patient T2 plasma and custom anti-GFAP demonstrates immunoreactivity to GFAP at 50kDa (Figure 21). These results are consistent with the findings from the LC-MS/MS showing SCI patient plasma immunoreactivity to GFAP.

Figure 21. SCI Patient Plasma Immunoreactive to Purified Recombinant GFAP Protein

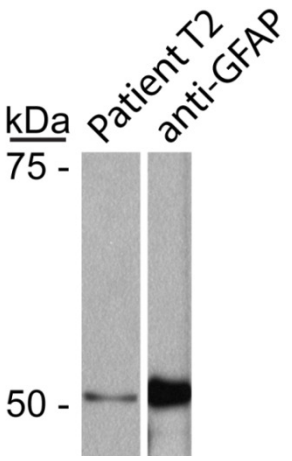


Figure 21. Representative lanes from a Western blot showing T2 with banding patterns consistent with positive immunoreactivity to GFAP protein. Lane 1 shows a SCI patient T2 plasma probed against purified recombinant GFAP protein. Lane 2 is a custom anti-GFAP antibody generated by the Dash Laboratory. These results are consistent with the LC-MS/MS results that identified GFAP as a potential antigen.

C. Capillary electrophoresis immunoassay and antigen verification validates the presence of GFAPab.

C.1 GFAPab is specific

The previous experiments suggest that the GFAP antigen, identified through 2-D electrophoresis and LC-MS/MS, produces immunoreactivity at T2 in some SCI patients. In order to estimate the proportion of the SCI subjects who are positive for GFAPab purified recombinant human GFAP protein was used as the sample on a capillary electrophoresis–immunoassay to measure the level of immunoreactivity produced by SCI patient plasma. Initial studies determined the plasma dilution and amount of protein to use for the assay. Figure 22 illustrates a SCI patient plasma's immunoreactivity to increasing amounts of GFAP (10, 20, 40, 80, 160 ng) and lack of immunoreactivity to a second protein (CRMP2) in the same amounts. Indiscriminant binding was not seen in this subject.

Figure 22 Immunoreactivity of a SCI plasma sample specific for GFAP in response to increasing amounts of GFAP protein and CRMP2 protein.

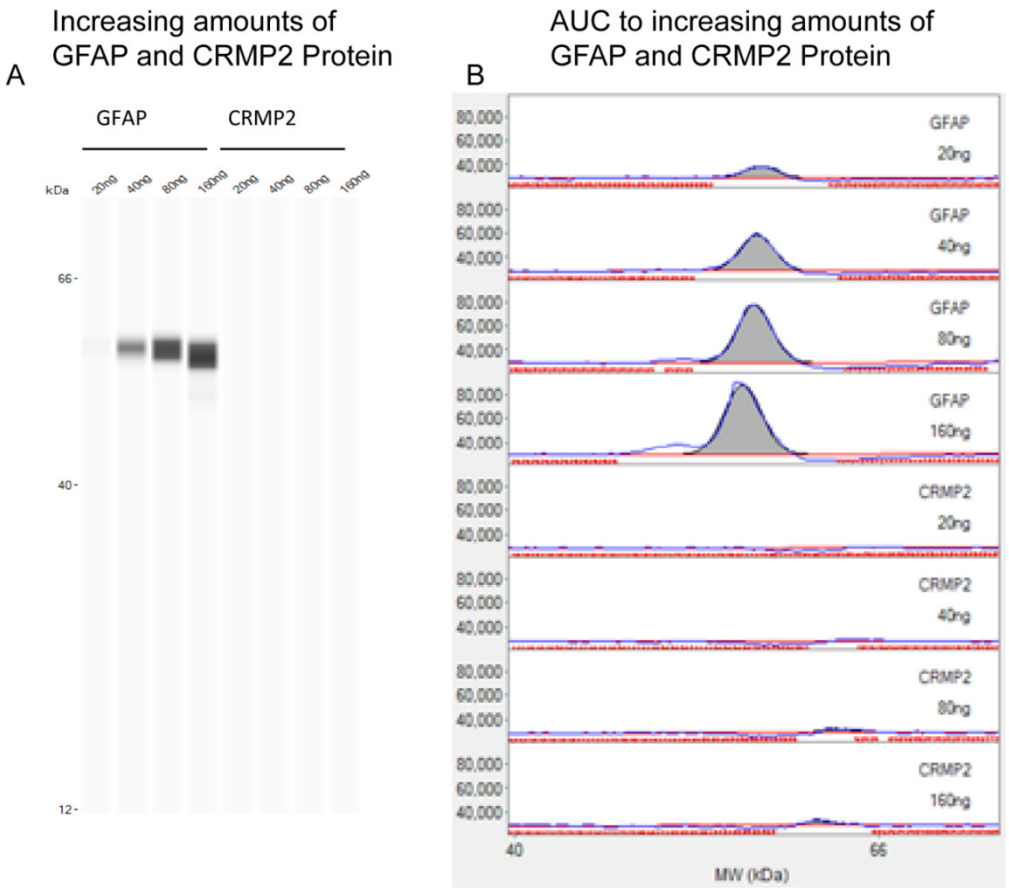


Figure 22 Immunoreactivity of a SCI plasma sample specific for GFAP in response to increasing amounts of GFAP protein and CRMP2 protein.

A) Purified recombinant GFAP protein was loaded in increasing amounts on lanes 1-4 (20, 40, 80, 160 ng), purified recombinant CRMP2 protein was loaded in lanes 5-8. Lanes were probed with human plasma (1:50) from a known GFAPab-positive subject and secondary anti-human HRP IgG. Immunoreactivity is seen in the GFAP lanes, but not in the CRMP2 lanes. This subject shows specificity for GFAP. For this subject the amount of GFAP is the limiting factor as indicated by the decreasing GFAPab area under the curve at lower amounts of protein.

To verify antigen-binding specificity, competition studies were performed using diluted plasma (1:50) by the capillary electrophoresis-immunoassay. Samples were incubated overnight at 4℃ either with or without 1600ng/10µl of the GFAP protein or an equimolar amount of a competing protein labeled with the same Myc-DDK tags (purified CRMP-2) to allow comparison between pre-absorbed samples with the same sample that underwent standard incubation. Figure 23 is a representative subject showing decreased immunoreactivity to GFAP after pre-incubation with GFAP. An immunoreactive area can be seen, corresponding to the molecular weight of GFAP. Immunoreactivity was reduced when pre-absorbed with GFAP whereas the pre-incubation with CRMP2 had no demonstrable effects on the immunoreactivity to GFAP protein. These results are consistent with the LC-MS/MS findings of GFAP as an antigen, and suggest the immunoreactivity to GFAP protein is specific.

Figure 23. SCI patient plasma immunoreactivity specific for GFAP.

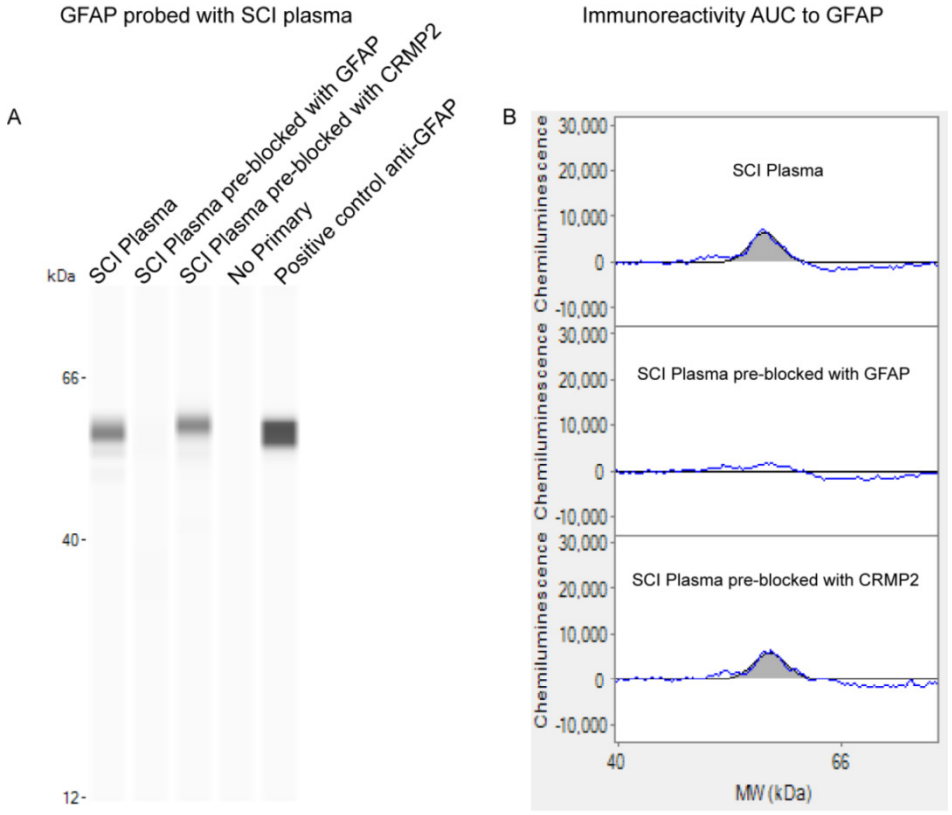


Figure 23. SCI patient plasma immunoreactivity specific for GFAP. A) Image showing SCI plasma immunoreactivity to GFAP protein (lane 1), plasma pre-blocked with GFAP (lane 2), plasma pre-blocked with competing protein (CRMP2) (lane 3), no primary showing no immunoreactivity (lane 4), anti-GFAP positive control (lane 5). Immunoreactivity from plasma is not seen in lane 2 that was pre-blocked with GFAP, but it is present in the plasma that was pre-blocked with CRMP2 showing the plasma antibody in this sample is specific for GFAP. B) AUC of plasma, pre-blocked plasma and competed plasma. C) Graphic representation of immunoreactivity change with pre-incubation of sample.

C2. GFAPab present in plasma samples

There was no statistically significant difference between GFAPab-positive and GFAPab-negative SCI patients based on sex, age, weight, complete/incomplete, or cervical/other level of injury.

For acute SCI patients, plasma samples collected 1.2 ± 0.7, 6.4 ± 1, 16 ± 7 and 96 ± 54 days after injury were assayed. Figure 24 presents the time course for GFAPab levels following SCI. There was a significant change in GFAPab over time ($F_{(3,37)} = 3.42$, $p=0.02$), with peak levels detected at the 16 day time point. For the 96 day sampling period, only 13 samples were available for testing. When assessed for the presence or absence of GFAPab at the 16 day time point, 21 of 38 (55%) acute SCI were found to be immunopositive. By comparison, 4 of 19 (21%) healthy volunteers were positive for GFAPab. The GFAPab levels measured at the 16 day time point were found to be significantly increased by comparison to those measured in healthy volunteers ($T=401.5$, medians 26,377 vs 0; $p=0.005$) (Figure 25).

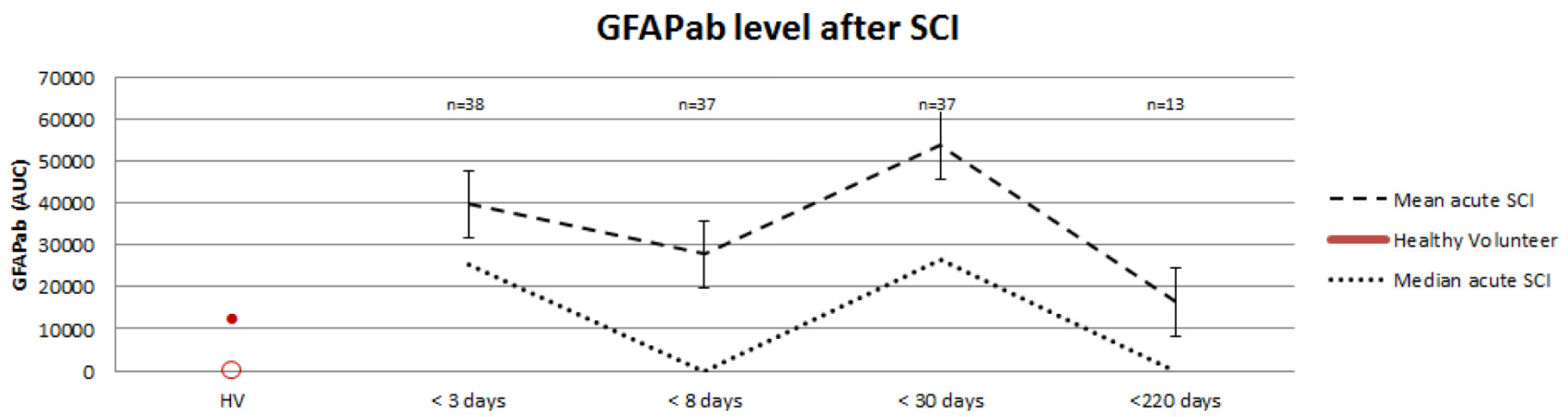


Figure 24 GFAPab levels after SCI over time

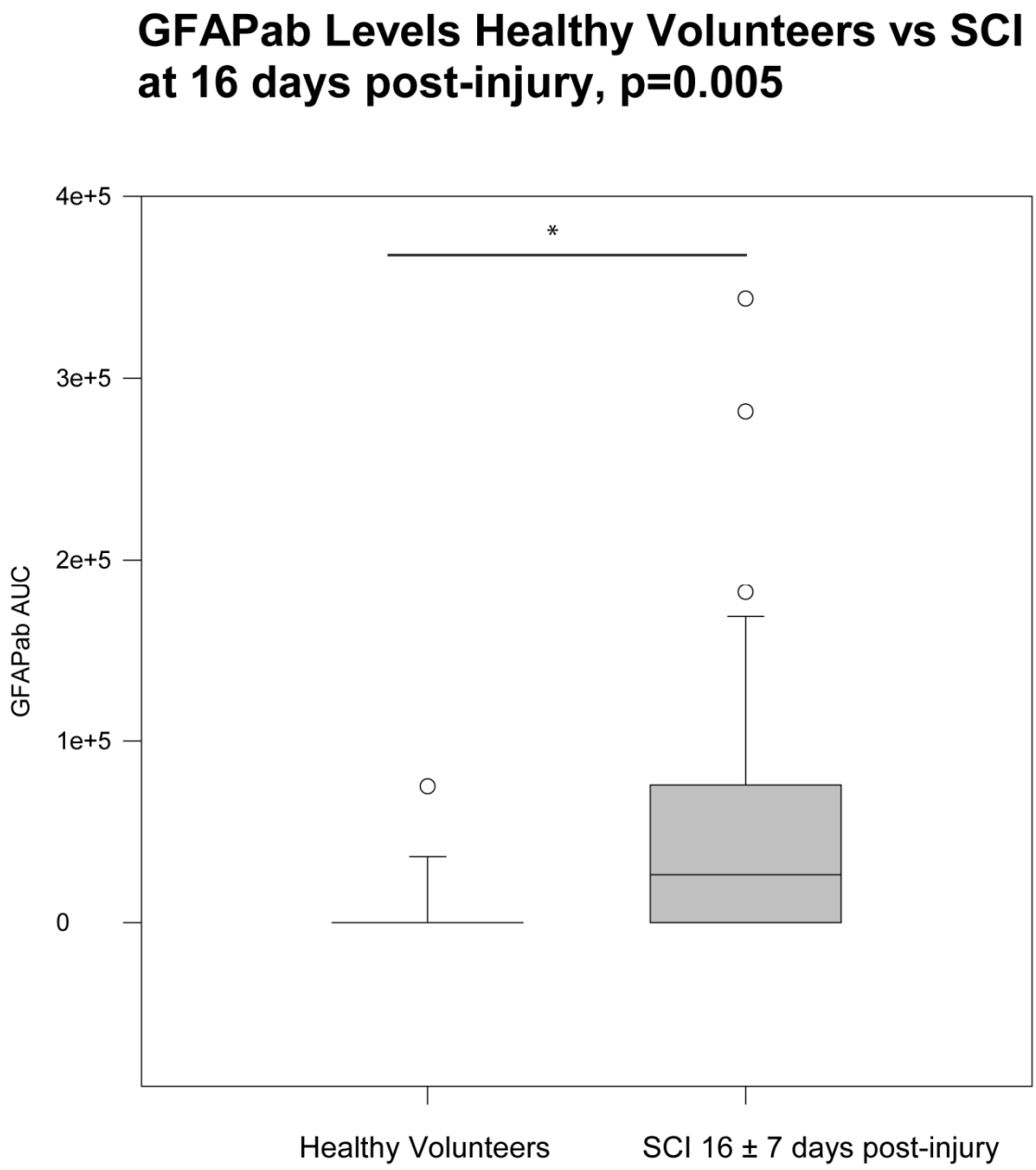


Figure 25. SCI patient plasma levels of GFAPab are significantly different from those of healthy volunteer controls. (T=401.5, medians 26,377 vs 0; p=0.005)

Samples from chronic SCI patients (1-41 years post-injury, mean of 15 years) were assayed for the levels of GFAPab. Of the 80 chronic SCI patients assayed, 34 (42.5%) were found to be immunopositive for GFAPab. When compared to the healthy volunteers, chronic SCI GFAPab levels were not significantly different (T=758, medians 0 vs 0, p=0.052; Figure 26). Sample size calculations based on data from theses chronic SCI and healthy volunteer subjects shows that 23,091 subjects would be needed to detect a significant difference at an 80% power with an alpha of 0.05.

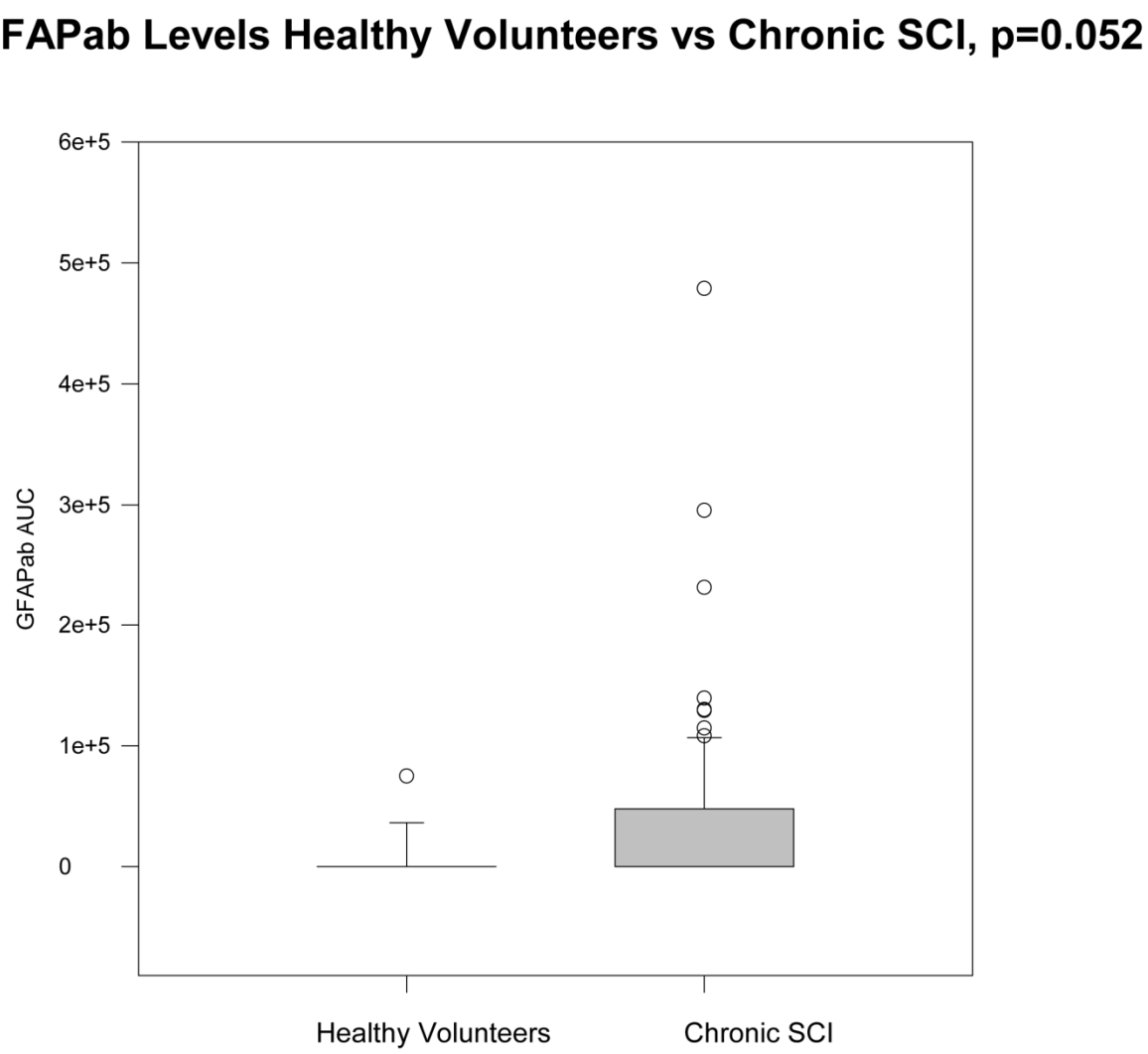


Figure 26. Chronic SCI patient plasma levels of GFAPab compared to healthy volunteer controls. (T=758, medians 0 vs 0, p=0.052; Figure 27). Sample size calculations based on data from theses chronic SCI and healthy volunteer subjects shows that 23,091 subjects would be needed to detect a significant difference at an 80% power with an alpha of 0.05.

C3. GFAPab in chronic SCI patients does not have diagnostic value for neuropathic pain.

Neuropathic pain was diagnosed in 46 (57.5%) chronic SCI subjects and they were classified as positive for neuropathic pain, whereas 34 (42.5%) of the chronic SCI patients were classified as not having neuropathic pain. GFAPab levels at the time of pain assessment could not distinguish between chronic subjects with or without neuropathic pain (T=1507, medians 0 vs 7898.5; p=0.16). When evaluated using a receiver operating characteristic (ROC) curve, chronic SCI GFAPab was found to have no diagnostic value (AUC = 0.42) in identifying chronic patients with neuropathic pain.

D. ROC curve analysis shows GFAPab levels distinguish acute patients who develop neuropathic pain versus those who do not.

In acute SCI patients, neuropathic pain was defined by a clinical diagnosis within 6 months of injury. Figure 27 shows GFAPab levels over time by pain group. Using the GFAPab levels measured at the 16 day time point, acute SCI subjects with neuropathic pain within 6 months had higher GFAPab levels than those without neuropathic pain (T=219, p=0.02) (Figure 28). There was a positive correlation between the presence of GFAPab at 16 ± 7 days and the development of neuropathic pain (r=0.46, p=0.003).

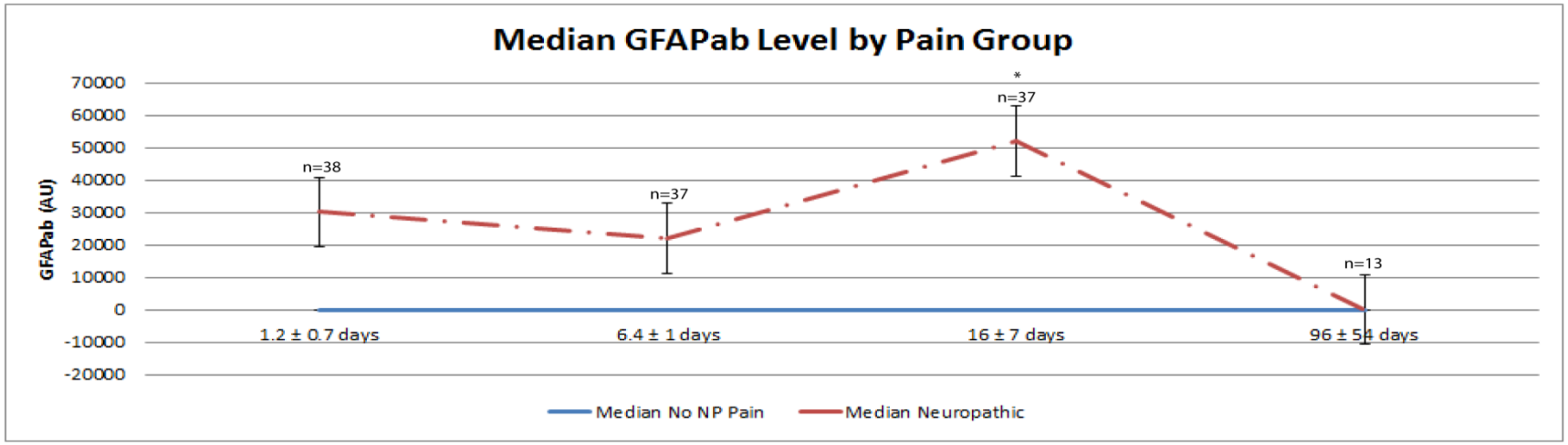


Figure 27. GFAPab level by pain group. The GFAPab level is significantly elevated at 16±7 days in those who develop pain within 6 months of injury (p=0.02)

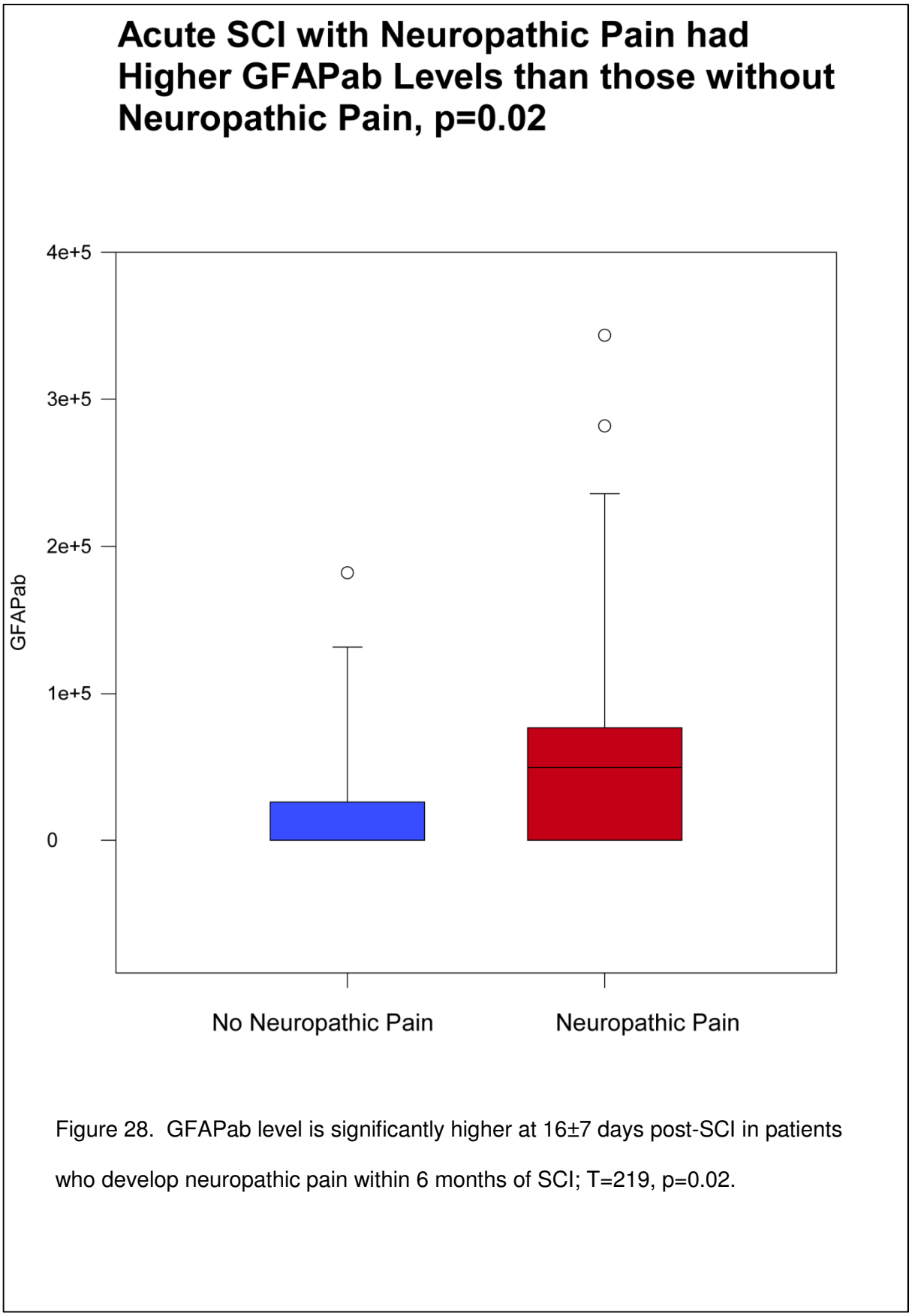
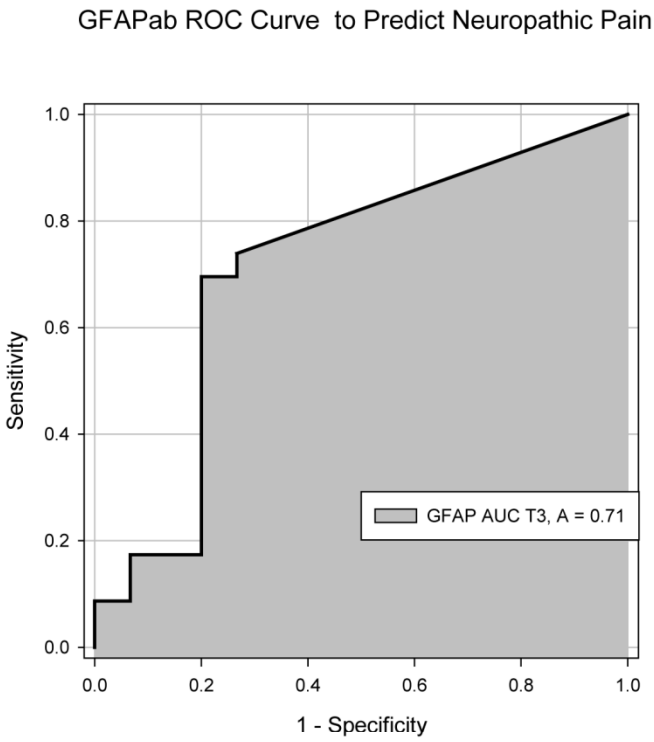


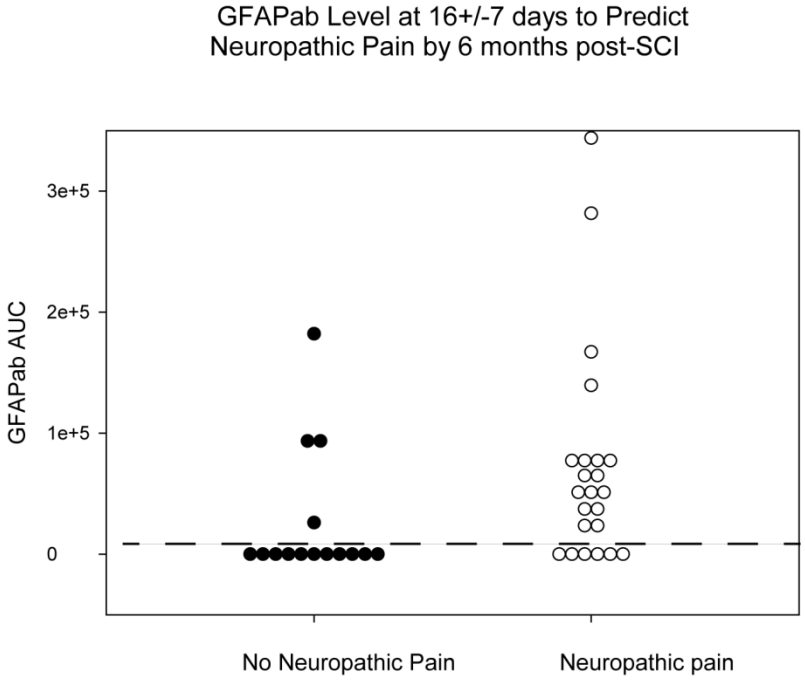
Figure 28. GFAPab level is significantly higher at 16±7 days post-SCI in patients who develop neuropathic pain within 6 months of SCI; T=219, p=0.02.

ROC analysis shows that GFAPab levels have a predictive value of 0.71 (95% CI, 0.53-0.89 p=0.03) for the development of neuropathic pain within 6 months after injury (Figure 29 A). Using a cut-off of 36 ng/mL (AUC=10,208) at 16 ± 7 days post-SCI, GFAPab had 74% sensitivity and 73% specificity for predicting neuropathic pain within 6 months of SCI (95% CI, 0.52-0.9 for sensitivity, 0.45-0.92 for specificity)(Figure 29 B).

Figure 29. Receiver operator characteristic analysis of predictive value of GFAPab for the development of neuropathic pain within 6 months of spinal cord injury.



A) Receiver operator characteristic analysis shows that GFAPab levels have a predictive value of 0.71 (95% CI, 0.53-0.89 p=0.03) for the development of neuropathic pain within 6 months after injury.



B) Using a cut-off of 36 ng/mL (AUC=10,208) at 16±7 days post-SCI had 74% sensitivity and 73% specificity for predicting neuropathic pain within 6 months of SCI (95% CI, 0.52-0.9 for sensitivity, 0.45-0.92 for specificity).

E. Predictive utility of GFAPab and CRMP2ab for the development of neuropathic pain after SCI.

These experiments identified two autoantibodies that are present in circulationg plasma of people with SCI. The peak immunoreactivity for the longitudinally collected samples was detected at 16 ± 7 days. Recognizing that no one autoantibody is likely to be the sole predictor of the development of neuropathic pain, the effect of the presence of the two autoantibodies on the the development of neuropathic pain was analyzed (Figure 30). A chi-square test was performed and a significant relationship was found between the presence of autoantibodies and the development of neuropathic pain, X^2 (1, N=38)=7.47, p=0.006. The presence of either or both antibodies at 16 ± 7 days post-SCI significantly increases the odds of developing neuropathic pain within 6 months by 9.5 times among those with SCI (95% CI, 2.08-43.50, p=0.006). When controlling for age, gender, body mass index, complete injury, and cervical level, the presence of GFAPab and/or CRMP2ab remains a significant factor in predicting neuropathic pain within 6 months with an odds ratio of 15.3 (95% CI 1.9 to 125, p=0.01).

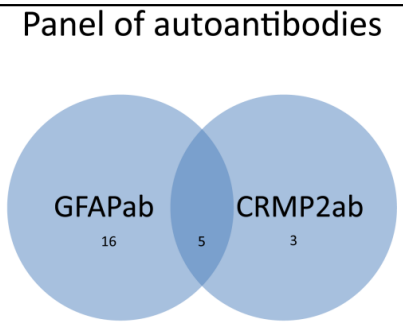


Figure 30. Panel of autoantibodies.

There were 24/38 (63%) of SCI subjects positive for one or both autoantibodies. A significant relationship was found between the presence of autoantibodies and the development of neuropathic pain, X^2 (1, N=38)=7.47, p=0.006. The presence of GFAPab and/or CRMP2ab increased the odds of developing neuropathic pain within 6 months of injury by 9.5 times.

4.4. Discussion

A. Key findings

1. GFAPab was present in 21/38 (55%) of acute SCI subjects, 34/80 (43%) chronic SCI subjects and 4/19 (21%) of healthy control volunteers.

2. The level of GFAPab in the acute SCI subjects was significantly higher than that of healthy volunteers and chronic SCI subjects with the 16 ± 7 day sample showing peak levels.

3. GFAPab levels in chronic SCI subjects were not significantly higher than GFAPab levels of healthy volunteers (T=758, p=0.052). It is possible that the timing of the acquisition of the sample, an average of 15 years after SCI, influenced the level of the GFAPab present in the plasma.

4. In chronic SCI patients, 46/80 (58%) had neuropathic pain at the time of sampling (1-41 years post-SCI). No difference in the levels of GFAPab could be detected in the chronic SCI patients with neuropathic pain compared to those without neuropathic pain.

5. There were significantly higher levels of GFAPab in the 16±7 day plasma of acute SCI patients who developed neuropathic pain (23/38, 60.5%) compared to those who did not develop neuropathic pain (15/38, 39.5%) within 6 months of injury and there was a positive correlation between the presence of GFAPab and the development of neuropathic pain.

6. When combining the presence of GFAPab with CRMP2ab at 16 days post-SCI the odds of developing neuropathic pain within 6 months was significantly higher (OR 9.5, 95% CI 2.08 to 43.50, p=0.006). This remained significant after controlling for age, gender, body mass index, complete injury, and cervical level (OR 15.3 95% CI 1.9 to 125, p=0.01).

B. There was more than one GFAP spot identified on 2-D gels

Seven of the spots sent for LC-MS/MS analysis were positive for GFAPab. These spots ranged in molecular weight from 38 to 50kDa and *pI* of between pH 5 to 6. Consistent with the LC-MS/MS findings (Methods 2.13 C), GFAP and its breakdown products have molecular mass of approximately 38-51kDa and a basal *pI* of pH 5.4. [192] The human CNS expresses 8 highly homologous GFAP isoforms (α, γ, δ/ε, κ, Δ135, Δ164, Δexon6, Δexon7).[169, 193] It is possible that the range of immunoreactivity seen in SCI patient samples represents recognition of different homologous isoforms. A recent study identified 22 of 451 (5%) patients with neurological autoimmune disease as having antibodies to GFAPα, 14 of these 22 patients were also immunopositive for GFAPδ.[194]

Ishida et al. performed 2-D western blotting analysis comparing immunostaining of patients with autoimmune dementia to vascular dementia patients and to a standard anti-GFAP antibody. A broad range of *pI*s existed in the patients' immunoreactivity, and the patients with autoimmune dementia had more alkaline (high) *pI*s than those of the commercial antibody or of vascular dementia patients' immunoreactivity.[195] The differences in *pI*s are related to the degree of phosphorylation, with higher phosphorylation, GFAP becomes more acidic.[195] Different patients may form autoantibodies to different post-translational modifications of GFAP. The Ishida et al. commercial anti-GFAP, the custom anti-GFAP and SCI patients in the present study showed immunoreactivity at the size of intact GFAP as well as to degradation products of lower molecular weights. The Ishida et al. patients' immunoreactivity ranged between 40-58kDa, similarly the spots identified as GFAP evaluated on patients in the present study ranged from 38 to 50kDa. Truncated GFAP at 38-48kDa in addition to the intact 50kDa protein on western blot has been reported and it was suggesting the immunoreactivity recognized GFAP that had undergone postmortem proteolysis in the CNS homogenate.[192] Calpain cleavage of

GFAP to 38kDa was reported in a TBI study which proposed that the 38kDa fragment was the primary autoantigen inducing an autoimmune response.[196]

C. Some healthy volunteers have GFAPab

Four healthy volunteers were found to have detectable GFAPab. All healthy volunteers represented themselves as healthy. A history of prior TBI and/or concussion was not recorded at the time of sample collection; therefore it is unclear if the levels of GFAPab seen in these patients were due to a previous, undocumented CNS injury, neurosurgical procedure or an ongoing inflammatory disease state.

A study of TBI patients who had autoantibodies to GFAP found 64.2% (34/53) of their TBI subjects 10 days post-injury and 15.2% (15/96) of healthy controls were positive for GFAP autoantibodies.[196] The TBI patients' serum GFAP level at 1 day after injury and GFAP autoantibodies 4 to 10 days post injury was correlated (p=0.048).[196] GFAP autoantibodies have also been identified in patients with neoplasms (breast, ovarian and brain (GBM) cancer and thymoma).[194] Additionally, GFAP autoantibodies were reported in diabetes type I and type II. [197, 198] There was no relationship found with GFAPab or neuropathic pain in chronic or acute SCI coupled with a diagnosis of diabetes in the present study; however, there were only one acute SCI and 5 chronic SCI subjects with diabetes. It is not known if any of the SCI subjects or healthy volunteers developed diabetes after the study ended. Diabetes studies evaluating the correlation of the presence of GFAPab with the development of diabetic neuropathy and neuropathic pain are unreported in the literature.

D. Potential role of GFAP autoantibodies in developing neuropathic pain

SCI causes a breakdown of the BSCB that allows for the infiltration of circulating cells and molecules into the injured cord, and also allows the efflux of cellular debris and proteins.[199, 200] Consistent with this, it has been previously reported that the astrocytic protein GFAP can be detected in the plasma of SCI patients.[201, 202] However, there was no correlation between neuropathic pain at 6 months and CSF GFAP protein levels at 24 hours.[202]

The peak levels of GFAPab occurred at 16 ± 7 days after SCI. It is possible that the early availability of GFAPab contributes to the development of neuropathic pain and that once its action has transpired, it may no longer be required to maintain neuropathic pain. Alternatively, GFAPab could contribute to a continuously permeable BSCB. The presence of antibody fostering ongoing inflammation could lead to a perpetually permeable BSCB and promote neuropathic pain.[25]

Chapter 5. Complement Components C3 and C5

5.1. Introduction

A. What is complement?

The complement system includes over 40 proteins that function together to defend the host against pathogens and infection through a coordinated cascade of processes that opsonize pathogens, clear host cells after apoptosis, clear immune complexes and enhance antibody response.[187] Complement is made primarily in the liver and circulates in blood in its inactive form. After SCI, activation of complement is initiated by the trauma to the tissue. In this context, complement activation is involved in clearing apoptotic cells and neuronal or glial cell fragments.[23] Activated components can be found at the injury site as well as in systemic circulation.[45]

There are three main complement pathways (classical, lectin and alternative) that converge at complement component C3. The classical pathway is initiated by the binding of complement C1q to immune complexes or to pathogen-associated molecular patterns (PAMPs) on pathogen surfaces which initiates a conformational change that activates C1r and C1s leading to cleavage of C2 and C4 resulting in C4b-C2a (the C3 convertase).[203] The lectin pathway detects PAMPS or damage-associated molecular patterns (DAMPs, on damaged host cell surfaces) through mannose-binding lectin (MBL) or ficolins which causes the mannose-binding lectin-associated serine proteases (MASPs) to activate C2 and C4 resulting in the formation of C3 convertase.[203] The alternative pathway is activated spontaneously through C3 hydrolysis. With C3 hydrolysis, C3b binds to complement factor B (FB) forming C3b-B which, when activated, is processed by complement factor D (FD) resulting in alternative pathway C3 convertase (C3b-Bb).[203] (Complement can also be activated through the coagulation cascade.[204])

The cleavage of C3 by C3 convertases leads to activation of C3a, opsonization and clearance of pathogens, and activation of C5. C5 is the target component which is cleaved by the C5 convertase complexes leading to C5a release and the initiation of the membrane attack complex (MAC) pathway which culminates in cell lysis and death. Normally, complement regulatory molecules present on host cells (e.g., membrane cofactor protein, or complement receptor 1) and plasma regulators (factor H) protect the host cells against complement activation against host cells.[187] (Figure 31, adapted from[187, 203, 205]).

Figure 31 Model of Complement Cascade

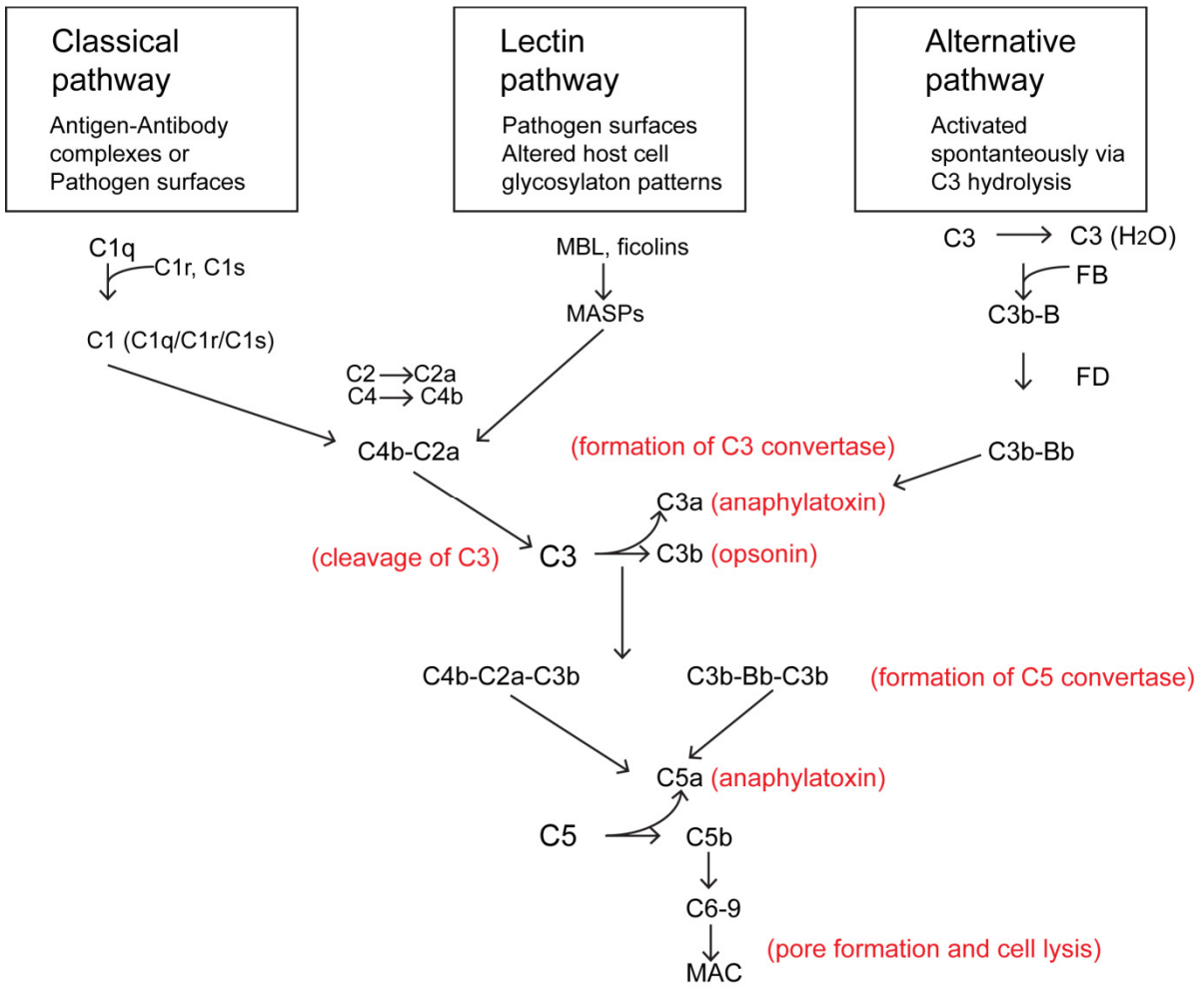


Figure 31. Model of the complement cascade. The complement cascade is an important host defense mechanism against infection and disease. There are three main activation pathways of the complement cascade, the classical, lectin and alternative pathways. The pathways have different initiators of activation; however they converge at complement C3. The cleavage of C3 by C3 convertases leads to activation of C3a, opsonization and clearance of pathogens, and activation of C5. C5 is the target component which is cleaved by the C5 convertase complexes leading to C5a release and the initiation of the MAC pathway which culminates in cell lysis and death. (MBL, mannose binding lectin; MASP, mannose-binding lectin-associated serine proteases; FB, complement factor B; FD, complement factor D; MAC, membrane attack complex.)

Complement anaphylatoxins C3a and C5a are chemotactic mediators that lead to the activation of immune cells.[203] C3a and C5a stimulate polymorphonuclear cells (e.g., neutrophils, eosinophils, and mast cells) to release histamine and arachidonic acid metabolites (prostaglandins, leukotrienes and lipoxins), all of which are critical in events occurring during tissue damage. The histamine release causes vasodilation, enhanced vascular permeability and endothelial activation; prostaglandins contribute to vasodilation, pain and fever; leukotrienes also increase vascular permeability, chemotaxis and leukocyte adhesion and activation; lipoxins act as antagonists to leukotrienes.[206] The increased vascular permeability and leukocyte and endothelial activation contribute to increased blood-spinal cord (BSCB) permeability after SCI.

Complement activation contributes to inflammation as part of the innate immune response and provides a bridge to the adaptive immune system through interactions with B- and T cells.[203, 207, 208] Complement C1q activates complement by binding to the Fc portion of surface-bound antibodies and immune complexes.[205] Complement C3 binds to circulating B- and T-lymphocytes and dendritic cells. Complement also produces pro-inflammatory effects through complement anaphylatoxins (C3a and C5a). [188, 209, 210] The combined presentation of complement receptors and B cell antigens enhances the activation of B cells.[211] Complement clears the immune complexes of B cell produced pathogen-specific antibodies and the pathogen antigen.[187]

B. Complement activation and neuropathic pain

Increased complement component concentrations have been associated with neuropathic pain, related in part to the pro-inflammatory effects of complement discussed previously. Chronic constriction of the sciatic nerve in a rat model demonstrated increased C3 mRNA expression in the spinal dorsal horn as measured by RT-PCR that was correlated with hyperalgesia ($r=0.899$, $p<0.0001$).[23] The levels of dorsal horn C3

mRNA increased over the 7 days evaluated and these increases corresponded with the development of hyperalgesia. C3 immunoreactivity was also increased by 2-fold and C3 concentration in spinal cord homogenate was up to 3.5-fold higher in the injured rats compared to sham controls. The level of circulating C3 was 300-fold greater than the CSF C3 level, suggesting that a breech in the BSCB allowed complement components to penetrate the BSCB, thus elevating the concentration of complement in the cord. GFAP (a marker for activated astrocytes) was also increased in the spinal cord in the injured rats with hyperalgesia.[23]

After SCI a positive feedback cycle is created from the elevated levels of complement accumulated at the injury site that stimulates the release of inflammatory mediators which then further activate the complement cascade.[187] The activated complement perpetuates increased vascular permeability, leukocyte recruitment and activation, pain and sustained tissue damage.

Using three distinct peripheral nerve injury models (chronic constriction injury, sciatic inflammatory neuropathy, intrathecal injection of HIV-1) Twining et al. administered an intrathecal complement inhibitor (soluble human complement receptor type 1) that blocked C3a, C5a and the formation of the MAC. Use of the complement inhibitor correlated well with the elimination of the mechanical allodynia that was induced in all three different peripheral injury models.[212] Griffin et al. performed a gene chip screen on RNA from dorsal horn in peripheral neuropathic pain rat models and identified an increase of genes expressing complement proteins (C1qb, C1qg, C4 and C3).[213] The relationships of complement components C5, C5a, C5b and C6 to pain sensitivity were evaluated. C5a was injected into the intrathecal space of uninjured rats which induced cold pain sensitivity; additionally, a C5a receptor antagonist injected into the intrathecal space of peripheral nerve injured rats resulted in decreased cold hypersensitivity.[213] C5-deficient mice with peripheral nerve injury had decreased pain sensitivity, whereas C6-deficient rats with

peripheral nerve injury (C6 is downstream of C5b) had no change in pain sensitivity leading to the conclusion that in this model C5a mediates neuropathic pain. [213]

Overall, these experimental models show a strong relationship of complement components C3 and C5 mediating the development of trauma-induced neuropathic pain.[23, 210, 212, 213] Data presented here indicate that after SCI some patients develop CRMP2ab and GFAPab and that there is an association between GFAPab at 16±7 days and neuropathic pain within 6 months of SCI. Complement and complement breakdown may be contributing to the association seen between the GFAPab and the development of neuropathic pain after SCI. Supporting this, impaired clearance of antigen-antibody complexes from deficiencies in complement increases the risk of developing an immune complex-mediated disease such as in systemic lupus erythematosus (SLE).[214, 215] Complement activation after SCI is expected. The physical damage to the cells is the cause of complement activation and complement infiltrates to the injury site.[216] Therefore, analyses were performed to test whether SCI results in a reduction in the circulating levels of complement C3 or C5 in human patients reflecting complement migration of C3 or C5 to the site of injury.

The overriding hypothesis is that complement components and their subsequent cascade related components contribute to the association between the GFAPab and the development of neuropathic pain. However, as results below show, a reduction in complement after the initial complement activation was not observed in the present study.

5.2. Methods

A. Plasma collection and patient classification

Plasma from consented acute SCI patients was collected as described in Method 2.3. Patients were classified according to the presence or absence of neuropathic pain as described in Method 2.2.

B. Enzyme-linked immunosorbent assay (ELISA)

Complement C3 and C5 levels were evaluated in the acute SCI plasma samples to determine whether there was an association with plasma levels of these proteins with the presence of GFAPab, CRMP2ab or the development of neuropathic pain. To evaluate levels of complement C3 and C5, enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's instructions (abcam ab108822 and ab125963). Plasma previously collected in EDTA tubes and frozen at -80◦C was diluted 1:800 for complement C3 and 1:20,000 for complement C5 assay. Each plasma sample was assayed in duplicate on 96-well plates. For the C3 assay plasma or standard were incubated for 2 hours with biotin followed by washing, then streptavidin-peroxidase conjugate was added to each well, incubated for 30 minutes and washed. For the C5 sandwich ELISA, sample or standard was added to each well and allowed to incubate for 2 hours. The plate was washed and then biotin was added and allowed to incubate for one hour. This was followed by washing; streptavidin-peroxidase conjugate was added to each well, incubated for 30 minutes and washed. After the last wash for both assays, tetramethylbenzidine (TMB) was added followed by the stop solution and the absorbance was read at 450nm with a correction at 540nm. The calculation of unknown concentrations from the standard curve was performed using Systat Software, Inc. SigmaPlot for Windows (San Jose, CA). The minimum detectable threshold of the Complement C3 assay is 0.2µg/mL and normal plasma levels range from 900 to 1900 µg/mL.[217, 218] The assay minimum detectable threshold of Complement C5 is 0.1ng/mL and normal plasma levels are 65µg/mL(abcam).[219, 220]

Statistical analysis was performed as described in Chapter 2.11.

5.3. Results

A. Complement C3 or C5 are activated after SCI

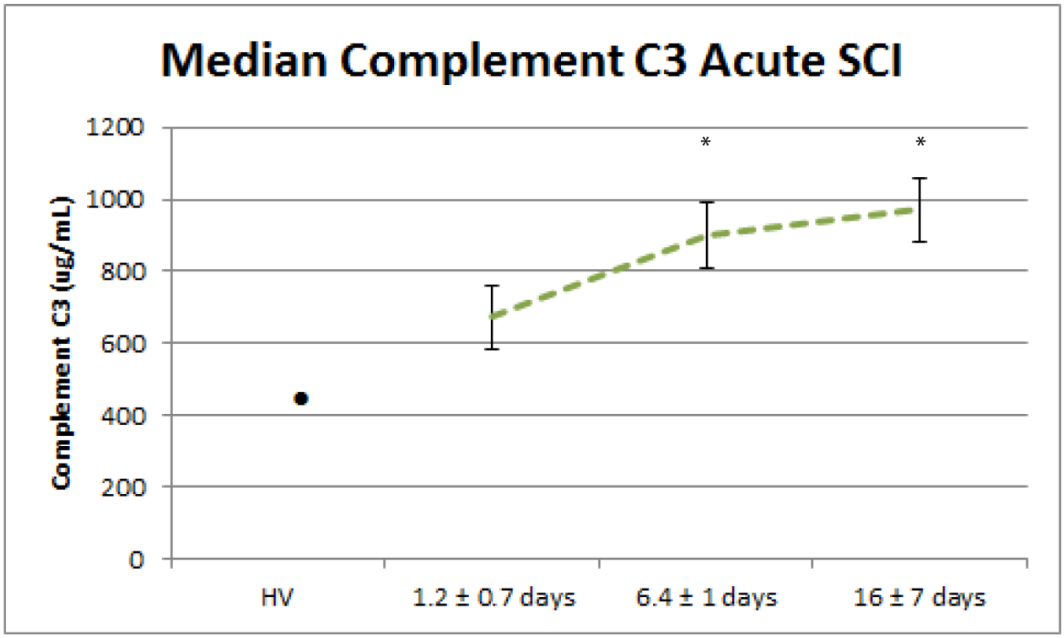
Plasma levels of complement C3 and C5 were measured using ELISAs. Median values for complement C3 were 472.3 µg/ml for healthy volunteers and 673µg/mL, 901.3 µg/mL and 970.9 µg/mL for 1.2, 6.4 and 16 days post-SCI, respectively. There was a significant increase in the C3 levels between 1.2 days and C3 levels at 6.5 and 16 days ($F(2,37)=34.1$, $P<0.001$). The levels of C3 at 1.2 days compared to healthy volunteers was not significantly different ($T=77$, $p=0.056$). At the 6.4 and 16 day time point, C3 was significantly elevated compared to healthy volunteers (6.4 day: $T= 32$, $p<0.001$; 16 day: $T=33$, $p<0.001$).

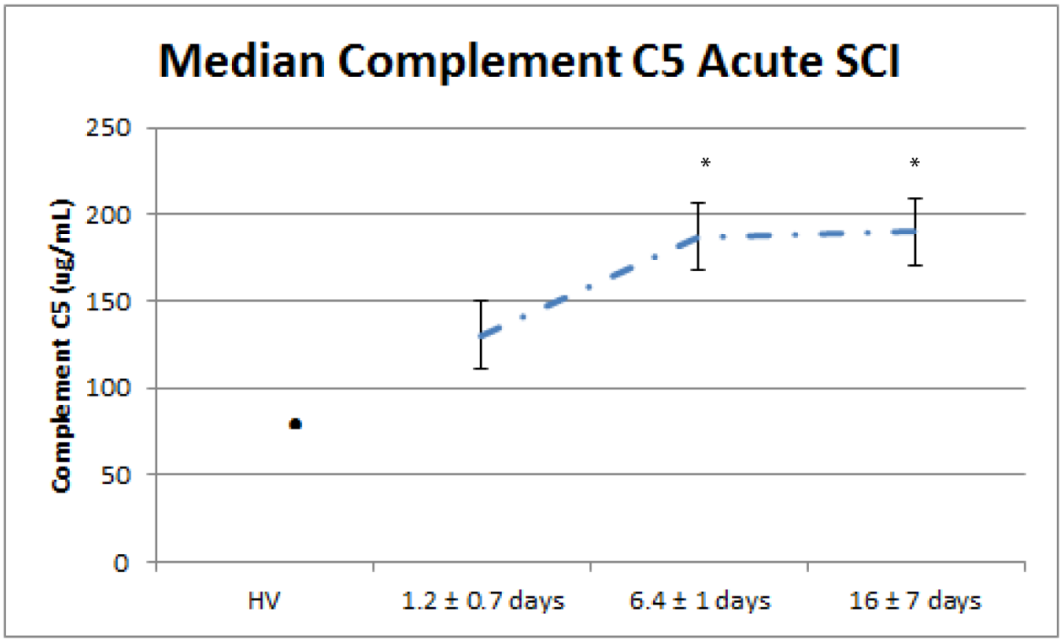
Median values for complement C5 were 96 µg/mL for healthy volunteers and 130µg/mL, 188µg/mL and 189.8 µg/mL for 1.2, 6.4 and 16 days post-SCI, respectively. There was a significant increase in C5 levels between 1.2 days and C5 levels at 6.5 and 16 days after SCI ($F(2,37)=36.7$, $p<0.001$). The levels of C5 at 1.2 day compared to healthy volunteer samples was not significantly different ($T=76$, $p=0.052$). C5 was significantly higher at the 6.4 and 16 day time points compared to healthy volunteer levels (6.4 day: $T=35$, $p<0.001$; 16 days $T=35$, $p<0.001$) (Figure 32).

B. A correlation is observed between the levels of complement C3 but not C5 with the levels of GFAPab and CRMP2ab.

Assessments were made to test whether there is a correlation between the levels of C3 or C5 and the levels of GFAPab or CRMP2ab at each time point. There is a positive correlation between the 6.4 day levels of complement C3 and GFAPab levels at 16 days

(r(34)=0.34, p=0.04), but not any other time point. There is a positive correlation between levels of C3 and CRMP2ab at 16 days (r(31) = 0.41, p=0.02), but not at any other time point.



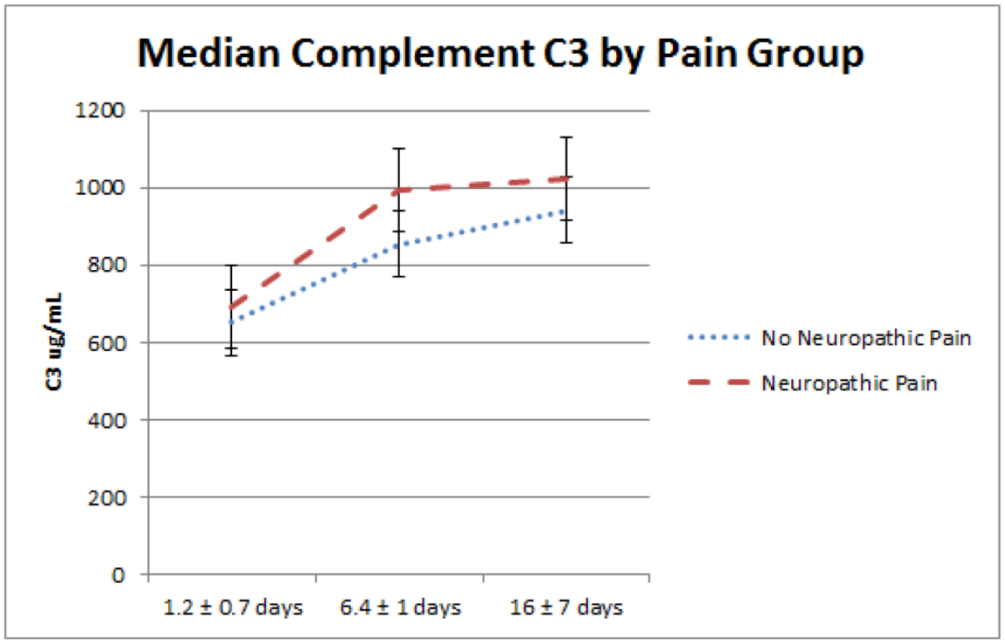


P<0.001 vs HV

Figure 32. Complement C3 and C5 levels in SCI patient plasma over time compared to healthy volunteer control levels. C3 and C5 are significantly elevated at the 6 and 16 day time points. C3: ($F(2,37)=34.1$, $P<0.001$); C5: ($F(2,37)=36.7$, $p<0.001$)

C. An association is not found between complement C3 or C5 with the development of neuropathic pain

Complement C3 or C5 levels are not different between those who developed neuropathic pain compared to those who did not (Figure 33). There is not a significant difference in levels of C3 by pain group $F(1,35)=0.48$, $p=0.49$ and there is not a statistically significant interaction between pain group and time $F(2,35)=0.004$, $p=1$. For C5 levels, there is also no significant difference in C5 levels by pain group $F(1,35)=0.49$, $p=0.49$, and there is not a statistically significant interaction between pain group and time $F(2,35)=0.58$, $p=0.57$.



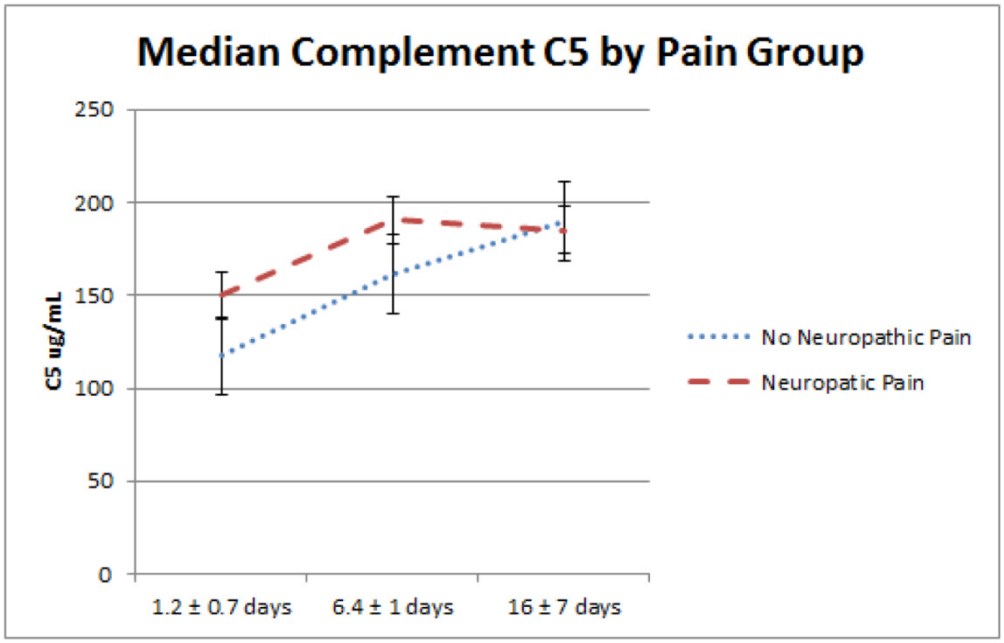


Figure 33. Complement C3 and C5 levels did not differ by pain group. There is not a significant difference in levels of C3 by pain group $F(1,35)=0.48$, $p=0.49$ and there is not a statistically significant interaction between pain group and time $F(2,35)=0.004$, $p=1$. For C5 levels, there is also no significant difference in C5 levels by pain group $F(1,35)=0.49$, $p=0.49$, and there is not a statistically significant interaction between pain group and time $F(2,35)=0.58$, $p=0.57$.

5.4 Discussion

A. Key Findings

Plasma levels of complement C3 and C5 were increased after SCI through the 16 day time point. Complement C3 and C5 levels did not differ by pain group.

B. Elevation of complement C3 and C5 after SCI.

Contrary to the hypothesis, circulating complement levels did not decrease, but rather increased over time after injury. It was hypothesized that circulating C3 and C5 levels would decrease as they migrated to the injury site. Alternatively, the upregulation of C3 and C5 may be the result of ongoing inflammation.

Anderson et al. evaluated the deposition of complement components C1q, C4, Factor B (binds C3b) and MAC (C5b-9) in the spinal cords of SCI rats experiencing mild or severe contusion injuries compared to laminectomy-only rats at 1, 7 and 42 days post-SCI.[216] These time points are similar to the time points assessed in the acute SCI human subjects' circulating plasma. The Anderson et al. study discovered complement components C1q, C4, FB, C5b-9 activated and peaked within one day, and complement remained immunoreactive in the injured spinal cord for 6 weeks post-SCI. There was uniform magnitude of complement component staining both rostral and caudal of the lesion epicenter (>20mm) in SCI rats, but no immunoreactivity in the laminectomy controls. Changes due to BSCB permeability in the lateral or ventral white matter were not observed until 14 days, yet the complement immunoreactivity was present in gray and white matter at all levels of the spinal cord within one day, thus the authors concluded that BSCB permeability was not the only source of complement migration and that local complement synthesis was likely to have been enhanced by inflammatory mediators.[216] In addition to circulating blood complement permeating the BSCB and complement being produced

locally in spinal cord astrocytes, microglia and neurons, complement is found in polymorphonuclear leukocytes (PMN). C1q, C3, and C5b-9 were associated with PMN infiltration near the epicenter of the spinal cord.[221] PMN were detected at two hours, peaked at 24 hours and could still be detected by flow cytometry analysis 6 months after SCI. Three days after SCI at least 70% of PMNs infiltrating the spinal cord were associated with C1q or C3 and 95% were associated with C5b-9.[221] A study of 34 acute trauma victims that evaluated circulating C3a and C3 levels at multiple time points up to 7 days post-injury found an association with higher circulating C3a levels and non-survival only at the sample taken within 37 minutes after injury ($p=0.008$) and the C3a/C3 ratio was higher in non-survivors than survivors at about 1 hour post-injury ($p=0.033$).[222] The level of activation, not the duration of activation was related to non-survival. The acute trauma study indicated that complement activation occured almost immediately after injury and peaked early. Based on the timing of peak complement levels in the above studies, it is possible that a dip in circulating complement would have been missed with the three time points measured here. Alternatively, the local production may have supplemented circulating levels to sustain complement levels.

C. No association found between complement C3 and C5 levels and neuropathic pain.

The circulating C3 and C5 levels in SCI human subjects were elevated during the 30 days measured; however, there is no association with the presence of neuropathic pain within 6 months. It is possible that the timing of the complement measurements were too remote from the later determination of neuropathic pain and that if complement was measured at 6 months in the neuropathic pain patients it may have been elevated compared to those without pain.

Chapter 6. General Discussion

A. Summary of findings

The primary finding from this investigation is that SCI patients who developed CRMP2ab and/or GFAPab at 16 days had 9.5 times greater odds of developing neuropathic pain than patients who did not develop these autoantibodies. Additional findings include: 1) 34/80 (43%) of chronic SCI patients had GFAPab. 2) Autoantibody levels peaked at 16 ± 7 days and returned to healthy volunteer levels by 96 ± 54 days after SCI. 3) At 16 ± 7 days after SCI 8/35 (23%) patients developed CRMP2ab and 21/38 (55%) patients developed GFAPab. 4) The presence of GFAPab at 16 ± 7 days after SCI is a predictor of the development of neuropathic pain. 5) C3 and C5 remained elevated through 16 ± 7 days after SCI.

Inflammation has been identified as a contributing factor to the development of neuropathic pain after SCI.[102, 113, 223] After SCI, inflammation is initiated by the physical injury and the local immune response. Trauma results in cell damage which triggers an innate immune response to activate the complement system.[216] Complement enhances the ability of antibodies and phagocytes to clear cellular debris and pathogens, and it promotes inflammation as C3a and C5a are activated.[187] C3a and C5a anaphylatoxins stimulate neutrophils, eosinophils, and mast cells to release histamine and arachidonic acid metabolites (prostaglandins, leukotrienes).[187] Histamine promotes vasodilation, enhanced vascular permeability and endothelial activation; prostaglandins produce vasodilation, pain and fever; leukotrienes promote vascular permeability, chemotaxis, leukocyte adhesion and activation. The increase vascular permeability, leukocyte and endothelial activation augmented by the mechanical damage from the injury results in a permeable BSCB. [187] Increased permeability of the BSCB promotes the

activation of resident CNS immune cells. Cellular debris is transported to lymphoid organs for processing by immune dendritic cells enabling lymphocyte activation and the opportunity to generate autoantibodies.[214] Supporting this, B cells were present in the area of the lesions after SCI in mice and are also found in the CNS in humans with multiple sclerosis, an autoimmune disease.[9, 224] Astrocytes and infiltrating monocytes supply factors (B cell-activating factor (BAFF), and proliferation-inducing ligand (APRIL)) that are required for B cell functions (survival, differentiation, and germinal formation) and contribute to clonal expansion of B cells in situ.[225] This creates a favorable environment for autoantibody production.[225]

Autoantibodies that develop as part of the immune response after SCI have been identified as contributing to enhanced tissue damage and poor outcome.[9] B cells have been demonstrated to be causative of SCI pathology through 1) improved functional, behavioral and tissue recovery from SCI in B cell knockout mice compared to wild type mice; and 2) that B cell-mediated neurotoxicity is cause by SCI rodent IgG as injection of SCI IgG into uninjured mice resulted in SCI-like pathology.[5, 9] Until now, the identities of autoantibodies associated with the development of neuropathic pain in human SCI have not been identified. The hypothesis is that proteins from the injured spinal cord released by SCI trigger autoantibody production, which can lead to the development of NP. GFAP and CRPM2 are two antigens identified as inducing immunoreactivity from SCI patient plasma samples.

SCI patients who developed CRMP2ab and/or GFAPab had 9.5 times greater odds of developing neuropathic pain than patients who did not develop these autoantibodies.

Antibodies found in human blood vary between individuals based on their lifetime of variable exposures to pathogens, autoantigens, and other antigens. Autoimmune diseases have been characterized by the presence of patterns of autoantibodies. A panel of autoantibodies is likely to be more powerful than a single autoantibody to supplement or predict the clinical diagnosis of neuropathic pain after SCI. GFAPab and CRMP2ab are two autoantibodies that combined show a 9.5 times increased odds of developing neuropathic pain after SCI and when controlling for injury factors. The injuries being complete and/or at the cervical level were not associated with the development of the two autoantibodies.

Systemic lupus erythematous (SLE) is a chronic autoimmune disease affecting multiple organs including the skin, heart, lungs, kidneys, joints, and nervous system. SLE is an example of an autoimmune disease that is diagnosed based on clinical findings that are supplemented with results from evaluations of panels of autoantibodies including antinuclear antibodies, anti-double stranded DNA and other autoantibodies. Antinuclear antibodies are approximately 90% sensitive, but not very specific for SLE whereas anti-double stranded DNA is over 95% specific.[226] The panel of autoantibodies for SLE balances sensitivity and specificity to enhance the ability to distinguish SLE from other autoimmune conditions. When the panel of autoantibodies is combined it provides data on which to determine the presence of disease. For neuropathic pain after SCI, additional autoantibodies may improve the sensitivity and specificity in order to distinguish who will develop neuropathic pain. If the current findings are validated this panel of autoantibodies could be used as a biomarker for early diagnosis.

Forty-three percent of chronic SCI patients have GFAPab

The presence of circulating GFAPab was found in 43% of chronic SCI patients. ISNCSCI scores, age, sex and weight were comparable for both autoantibody- positive and

negative chronic SCI patients indicating the populations were similar. However, it is unclear why only a portion of chronic SCI patients generated GFAPab while others did not. It is possible that the autoantibody negative patients may not have been exposed to plasma GFAP levels sufficient to mount an immune response (either as primary or secondary exposure). When evaluating the levels of GFAPab in chronic SCI a difference in the levels of GFAPab between the pain groups was not detected. The chronic patients ranged from 1 to 41 years post-SCI. It is possible that the autoantibody-negative patients produced antibodies at other times. For example, blood levels of IgG antibodies in humans after receiving a tetanus toxoid boost increased by a factor of 80 between days 5-8 after injection then decreased after about 2 months reflecting production of short-lived plasma cells.[227] Short-lived plasma cells have an approximate half-life of human IgG of about 20 days, and long-lived plasma cells have a half-life of about 40 days.[227] Memory B cells can be antigen-dependent resulting in extensive proliferation and differentiation toward short-lived plasma cells, or polyclonal responding to polyclonal activators where all memory B cells respond undergoing continuous proliferation and differentiation maintaining a steady state.[227] The chronic subjects enrolled in this study were excluded if they had known ongoing inflammation (e.g., infection, cancer, skin breakdown, deep vein thrombosis), and they were on average 15 years post-SCI. These exclusion criteria may have reduced the ability to measure both short term serological memory (antigen-dependent) and long-term serological memory (antigen-independent polyclonal activation and differentiation of memory B cells).[227] It is possible that their GFAPab levels were below the assay's limit of detection at the time that their sample was acquired, years after autoantigen exposure.

The number of active memory cells is influenced by stimulation from cytokines (e.g., IL-2, IL-10, IL-21).[228-230] Antigen-specific immune responses directed at other non-cross reacting antigens impact the level of these cytokines, which then effects the volume

of active memory cells.[231] IL-2, IL-10, anti-CD40 and IL-21 induce plasma cell differentiation from memory B cells, and levels of these cytokines are higher in times of active inflammation.[230, 232-235] Chronic SCI subjects were excluded if there was a known, active ongoing inflammatory process. It is possible that excluding those subjects created a subject selection bias reducing the percentage of chronic SCI with perceptible GFAPab levels.

Autoantibody levels peaked at 16 ± 7 days and returned to healthy volunteer levels by 96 ± 54 days after SCI.

On exposure to an antigen lymphocytes with the antigen-specific receptor are activated and proliferate. Over a period of about 5 days B lymphocytes go through clonal expansion where they differentiate into effector cells.[179] The differentiated plasma B cells secrete antibody into the circulating blood as a response in order to clear the antigen.[236] Most plasma B cells die after the antigen is eliminated, but some of these antigen-specific B cells remain as memory cells and can be activated quickly.[237] For SCI subjects, the GFAPab secreted through activation of the memory B cells would require antigen stimulus to progress though the clonal expansion process.[227, 236] The peak levels of antibody detected in these samples were found to be at the 16 ± 7 day time point. These peak levels were generally higher than those observed in samples collected during the chronic stage of injury. Of exception, four of the chronic SCI subjects with levels of GFAPab that were more comparable to that seen acutely after SCI had evidence of disruption of the BSCB (e.g., recent placement of intrathecal pumps), suggesting a re-exposure to antigen may be responsible for the increased GFAPab levels observed in these patients.[227] Three of these four patients had neuropathic pain.

At 16 ± 7 days after SCI 8/35 (23%) patients developed CRMP2ab and 21/38 (55%) patients developed GFAPab.

Although the exact time course of GFAP release in humans after SCI has not been established, GFAP levels can be detected in the serum of traumatic brain injury patients within hours of the injury, returning to baseline by 3 days post injury.[174] In rat cortical and hippocampal brain tissue after controlled cortical impact CRMP2 decreased from 6 hours to 3 days, returning to normal levels by 5 days; the CRMP-2 55kDa breakdown products showed a corresponding increase over this time period.[156] Circulating levels of CRMP2 were not provided, but this breakdown of CRMP2 indicates that substrate could be available for autoantibody production after injury. Samples were not available for most patients within the very early time post-SCI in order to measure early release of GFAP or CRMP2.

After SCI astrocytes become reactive astrocytes. These reactive astrocytes have increased size, increased GFAP expression and increased number.[238] Recent analyses of rat tissue, CSF and serum showed release of GFAP protein and its breakdown products after SCI that was measurable at 4 hours, 24 hours, and 7 days post-SCI. Elevated GFAP/ breakdown product levels persisted at the injury site and in the serum longer than in the CSF. There was a positive association with injury severity and CSF and serum levels of GFAP/breakdown product.[239] The presence of GFAP/breakdown product after injury would facilitate the development of an autoantibody. In a TBI study, plasma taken within 24hrs of injury from TBI patients was compared with age-matched controls.[240] A new, specific IgG autoantibody response would not be expected to manifest this close to time of injury. In agreement with this, it has been reported TBI subjects who had previous TBI with loss of consciousness had significantly higher levels of GFAPab compared to controls, but TBI patients who had not had previous injury did not have a significant difference from

controls at that early time point. [240] This is one example of why some subjects may have antibodies to GFAP within a day of injury.

Twenty-three percent of SCI subjects had CRMP2ab at 16±7 days after SCI. These results did not demonstrate significance for an association of CRMP2ab with the development of neuropathic pain at this peak immune response time point (T=231.0, p=0.08). The subjects who were CRMP2ab positive with neuropathic pain represented 88% of CRMP2ab positive subjects and the CRMP2ab negative subjects who had neuropathic pain were 48% of CRMP2ab negative subjects. Sample size analysis based on these data indicates a larger sample size of 64 subjects is needed to detect a significant difference at an 80% power with an alpha of 0.05.

CRMP2 is a cytoplasmic protein found in central and peripheral axons.[155] CRMPs participate in semaphorin-induced growth-cone collapse and influence the direction of axon growth, dorsal root ganglion neuron growth, dendrite specification, and axon regeneration.[138] It is unknown what significance the presence of the CRMP2ab has in chronic SCI. It is possible that the presence of CRMP2ab could impinge on CRMP2's role in growth-cone collapse. Whether and how CRMP2ab is involved in inhibition of axonal guidance after SCI is of interest for future studies. The CRMP family of proteins includes CRMP 1, 2, 3, 4 and 5. CRMP1-4 have high sequence homology (approximately 75%).[138] The homology may have contributed to some of the nonspecific binding that was observed. No correlations were discovered with presence of CRMP2ab and level of injury, completeness of injury, presence of comorbid conditions, skin ulcers, spasticity, sex or age in *post hoc* analysis in the acute subjects. Five percent of healthy volunteers had CRMP2ab, this is of unknown significance. Autoantibodies to CRMP-2 have been identified in autoimmune retinopathy and cancer-associated retinopathy (melanoma, breast cancer and lymphoma).[155] None of the enrolled subjects were known to have retinopathy or cancer.

The presence of GFAPab at 16 ± 7 days after SCI is a predictor of the development of neuropathic pain.

Using a threshold of 36ng/mL of GFAPab in plasma has 74% sensitivity and 73% specificity for predicting the development of neuropathic pain within 6 months of SCI. GFAPab was not found to be diagnostic of neuropathic pain in the chronic patients, but data indicate that higher levels of in GFAPab in the acute stage it is a fair predictor.

One potential method of preventing the development of GFAPab would be to inhibit GFAP production and release into the circulation. There are medications that attenuate neuropathic pain in rodent models that inhibit glial cells activation, propentofylline, Ibudilast, and withaferin-A.[110, 241] Propentofylline inhibits GFAP production and was shown to reduce neuropathic pain behaviors in part through its regulation of pro-inflammatory cytokines.[110] Propentofylline and ibudilast inhibit phosphodiesterase and inhibit glial cell activation which has been correlated with reduced pain. [110] However, in human pain studies ibudilast did not have a positive effect in an opioid-overuse headache trial,[242] and propentofylline did not relieve pain in a post-herpetic neuralgia study.[243] Withaferin-A or ashwagandha is a steroidal lactone that binds and inhibits GFAP that was shown to have analgesic effect in rats.[192] Additionally, reduction in GFAP protein expression from clomipramine, a tricyclic antidepressant, was confirmed in mouse astrocytes after it was identified via a screen of chemical libraries.[244] Clomipramine, has been shown to reduce neuropathic pain in humans.[245]

C3 and C5 remained elevated through 16 ± 7 days after SCI.

Plasma levels of complement C3 and C5 levels were elevated after SCI through the 16 day time point. Serum complement elevation has been shown previously in human SCI

at 2 weeks to 9 years post-SCI.[24] Complement is constitutively present in the systemic circulation in its inactive form. Complement is activated early following trauma as part of the initiation of the inflammatory cascade. C3a and C5a are released systemically after complement system activation and exert pro-inflammatory effects. An increase of complement C3 genes is characteristic of reactive A1 astrocytes (reactive A2 astrocytes are neuroprotective and do not express C3); A1 astrocytes induce toxicity to synapses and contribute to death of neurons and mature oligodendrocytes after acute CNS injury.[162] After SCI, complement components are deposited in astrocytes, neurons, oligodendrocytes and axons of the injured cord.[45] In mouse injury models, C3 was found at the injury site within one hour peaking at 24 hours post-contusion.[246] Interestingly, C3 deficient mice had better locomotor scores than wild-type contused mice ($p<0.001$) suggesting C3 has a negative impact on motor function recovery. Additionally, there was more tissue preservation and less demyelination in spinal cords of C3 deficient mice. Here, the determination of the presence of neuropathic pain was based on pain development within 6 months and therefore acute measurements of circulating complement in the early post-injury time period may not directly correlate. Elevated complement may be one piece of the diagnostic puzzle to indicate which individuals develop neuropathic pain.[22, 213] However, the assumption that sequestration of complement due to GFAP or CRMP2 breakdown would be reflected as lower plasma C3 or C5 levels was not supported by the data. An alternate explanation could be that trauma to the tissue caused an activation of complement as part of an inflammatory reaction.

B. Weakness

There are several limitations to this study. First, the samples were collected at pre-determined time periods, therefore peak levels may not be represented for each subject,

and were likely not present in the chronic samples. There were 118 SCI patients to study, but only 38 of them with samples available at the time observed to be the peak for the group autoantibody levels. A larger sample size could strengthen these results as indicated in the power analysis performed using the CRMP2ab results discussed above.

Secondly, these studies are correlative and conclusions cannot be made on the nature of the presence of autoantibody causing neuropathic pain. If the presence of GFAPab or CRMP2ab is found to be causal, removal of the autoantibody could be explored as a preventive therapy. The effect of an autoantibody to GFAP or CRMP2 may be from a direct antibody-antigen binding causing protein misfolding, or functional interference with GFAP or CRMP2, or causing a decrease in availability of the GFAP or CRMP2 protein. Spinal cord tissue pathology cannot be determined based on these studies. Or the autoantibody may be contributing to pathology through increasing inflammation. Neuropathic pain may be caused by sprouting of pain fibers, hyperexcitability, sensitization, chronic BSCB permeability and persistent inflammation.[25, 103, 109] The presence of GFAPab and CRMP2ab may have contributed to the development of neuropathic pain by supplementing any of these processes. Astrocytes play an essential role in maintaining the BSCB. If GFAPab binds astrocytic GFAP it would likely disrupt astrocytes function which could contribute to a perpetually permeable cord or a change in the cytokines and immune cells migrating across the BSCB; this inflammatory state could lead to neuropathic pain.[25, 160] If the GFAPab and CRMP2ab are formed in response to proteins released from cells damaged after injury, it is possible that autoantibody-induced damage occurs and once a threshold of nerve damage and hyperexcitability has been reached the presence of the autoantibody is no longer necessary for neuropathic pain to persist.

Third, while validation studies have confirmed the GFAPab and CRMP2ab specificity, it is possible that the autoantibody after SCI may also be polyspecific. The

autoantibody may cross-react with the GFAP or CRMP2 antigen; however, it may have been developed in response to another agent. Multiple examples can illustrate polyspecificity, for instance, rheumatic fever is caused by anti-streptococcal antibodies (produced after an infection) that cross-react with a cardiac tissue antigen.[247] Antibodies to the Epstein-Barr virus have been shown to cross react with human heterogeneous nuclear ribonucleoprotein L (a protein involved in the formation and function of mRNA) related to identity of stretches of glycine-alanine repeats.[248] Homology exists between AQP4 and *C. perfringens* and overexpression of *C. perfringens* has been found in the intestine of NMO patients, indicating it could behave as a molecular mimic.[170] Molecular mimicry between *Campylobacter jejuni* which causes enteritis and ganglioside GM1 causing cross-reacting antibodies is involved in the development of Guillain-Barre syndrome (a demyelinating disease); anti-ganglioside GM1 has been identified in SCI.[16, 19, 249] These bacteria are found in the intestine, and GFAP is expressed in enteric glial cells. Enteric glial cells have a response similar to that of activated astrocytes under inflammatory conditions. They respond with an upregulation of GFAP, proliferate and hypertrophy; additionally they propagate immune signaling by secretion of IL1β, IL6 and TNFα, and expression of MHC class II molecules.[250]

In patients following trauma or severe injury intestinal bacteria can translocate through the disrupted gut barrier and induce systemic infection.[251-253] Mouse models have shown that pathological changes in the composition of the gut microbiome ("gut dysbiosis") cause immune dysregulation, neuroinflammation and exacerbate neurological disorders.[170, 254-257] A SCI mouse study that induced gut dysbiosis via the delivery of broad-spectrum antibiotics demonstrated that SCI-induced gut dysbiosis is associated with gut-associated lymphoid tissue immune cell activation, and that dysbiosis hinders recovery and worsens intra-spinal inflammation.[50] However, neuropathic pain was not evaluated in these gut dysbiosis SCI mice. The intestinal microbiota of 30 complete SCI patients was

compared to 10 healthy controls revealing that SCI patients had lower bacterial DNA counts of numerous butyrate producing bacteria.[258] However, symptoms of infection, inflammation or neuropathic pain were not evaluated. Butyrate producing bacteria play a key role in maintaining gut barrier function.[258]

Homology between common intestinal bacteria and GFAP and CRMP2 was explored as part of the present study. BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine) searches were performed for GFAP or CRMP2 looking for homology to some specific, common intestinal bacteria such as *Campylobacter jejuni*, *Helicobacter pylori* (both known to have structures that are associated with the development of autoimmune conditions[259]), *Escherichia coli*, *Enterobacter*, and the *Campylobacter* group. While an exhaustive search was not performed, it is clearly evident that CRMP2 shares high homology to bacteria in the *Enterobacter* family, suggesting molecular mimicry may be involved with the response observed in the acute subjects. GFAP shares homology with bacteria searched, but to a much lesser degree. For instance, when searching the *campylobacter* group, *dihydroorotase* [*Campylobacter concisus*] which is bacteria commonly associated with irritable bowel disease, results indicated it has 43% homology with 28% identity to CRMP2. A refined search to evaluate only the conserved domains of CRMP2 (to increase the chance of homology) did not reduce the area to be searched for CRMP2. GFAP conserved domains include the filament head domain (4-66) and the filament (68-376). Searching by the conserved domain of GFAP increased the percent homology. For example, searching the head domain against *Enterobacteriaceae* resulted in 65% homology with 61% identity. This gram negative bacteria family includes *Salmonella*, *E. coli* and *Klebsiella*. These results indicate that there is the possibility that the responses seen in plasma may be related to homology to pathogens to which the patient was previously exposed. Alternatively, concomitant inoculations with these pathogens could

have contributed to autoantibody formation. Regardless of the whether or not the autoantibody is polyspecific, it does not negate the fact that the presence of the autoantibodies at the 16 day time point showed a 9.5 times increased risk of neuropathic pain.

C. Model

The model suggests that the injury to the spinal cord causes damage to neurons and axons, and that astrocytes and microglia are activated, CNS proteins are released into the circulation and inflammation occurs. There is increased vascular and BSCB permeability and complement is activated exacerbating inflammation. Activated B cells generate autoantibodies; these autoantibodies (CRMP2ab and GFAPab) may interfere with normal cell function, damage tissue and perpetuate inflammation resulting in neuropathic pain (Figure 34, 35).

Figure 34. Model of the presence of autoantibodies at the SCI injury site

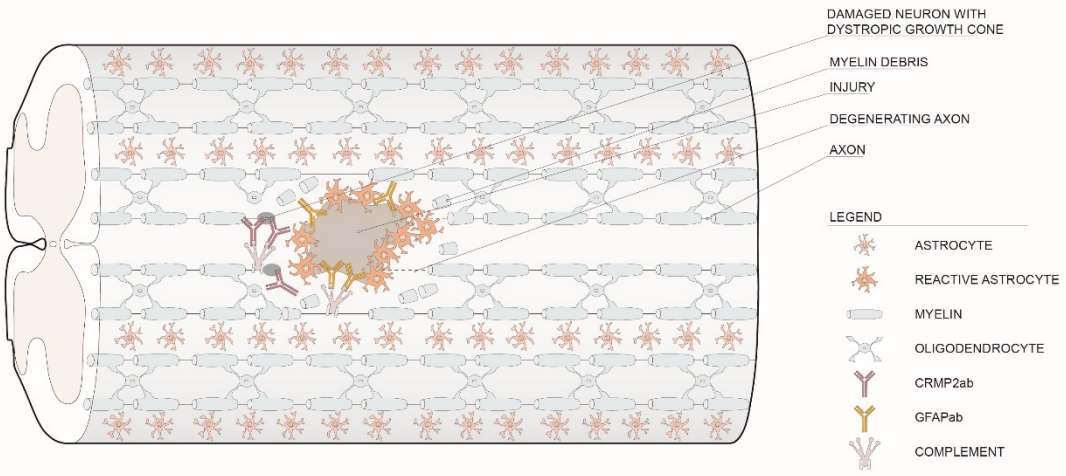
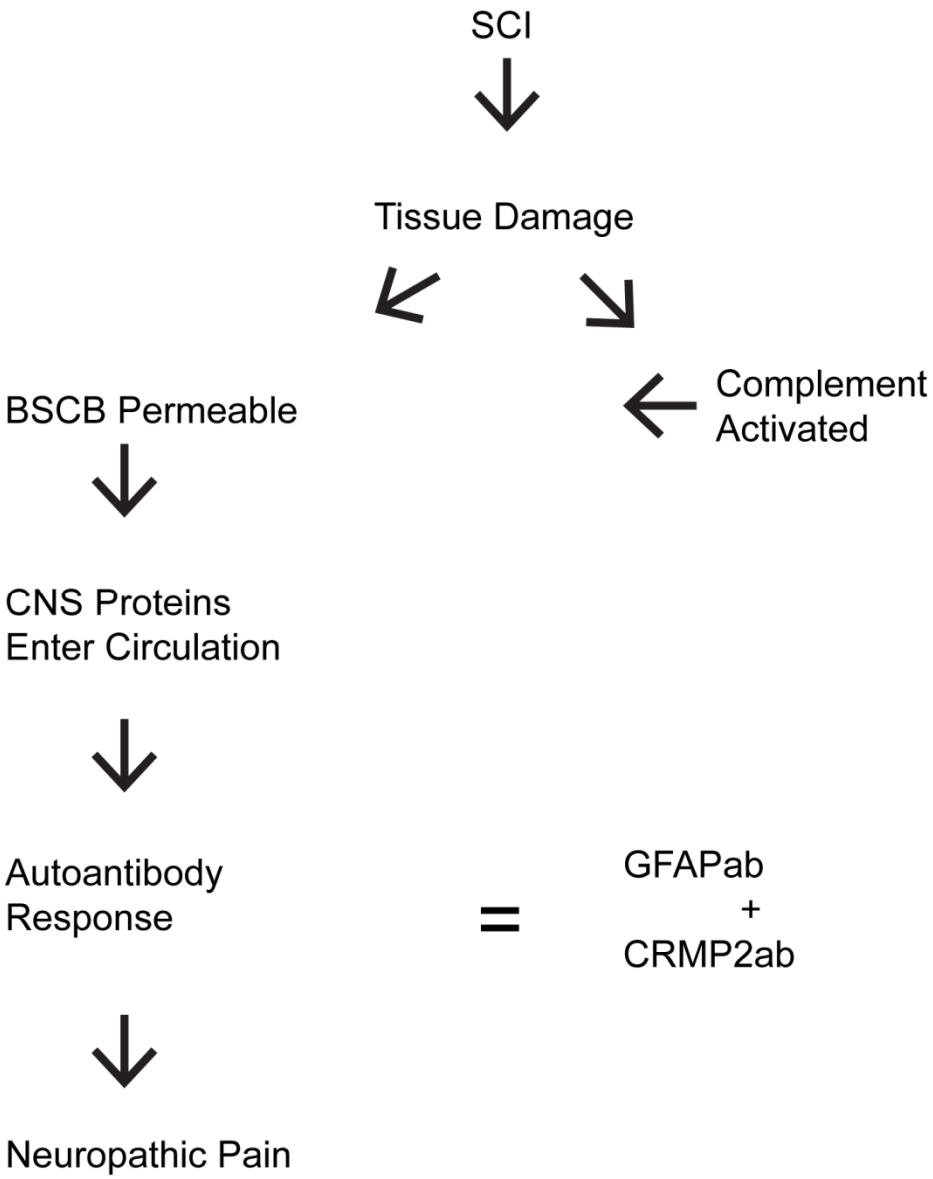


Figure 34. Model of the presence of autoantibodies at the SCI injury site.

The injury to the spinal cord causes damage to neurons and axons, astrocytes and microglia are activated, CNS proteins are released into the circulation and inflammation occurs, there is increased vascular and BSCB permeability and complement is activated exacerbating inflammation. Activated B cells generate autoantibodies; these autoantibodies may interfere with normal cell function, damage tissue and perpetuate inflammation resulting in neuropathic pain.

Figure 35. Overview Model



This research makes two contributions. It established a process for identifying autoantibodies and it identified two autoantibodies that are associated with the development of neuropathic pain within 6 months after SCI. The work established a process for identifying autoantibodies in human plasma. Using an unbiased approach, patterns of change in immunoreactivity produced by SCI patients' plasma were identified by western blot. The potential antigens were separated using 2-D gel electrophoresis. Areas with new or enhanced immunoreactivity were isolated, the corresponding gel spots were excised and the proteins were identified using LC-MS/MS. The antigen candidates were carefully confirmed as autoantigens causing the immunoreactivity. Critically, the capillary immunoassay blocking and competition studies revealed that non-specific binding can occur. The identification of non-specific binding demonstrates the utility of using this method over other methods which might not distinguish the specificity of binding.

The data indicates that there is potential for a diagnostic tool to differentiate patients who may develop neuropathic pain within 6 months after SCI from those who do not by assaying levels of GFAPab and CRMP2ab in combination with complement and other potential factors**.** Additionally, autoantibodies are likely to be present that are associated with other secondary pathologies. The identification of autoantibodies produced after SCI may lead to new treatment targets or new prognostic indicators.

D. Future directions

GFAPab and CRMP2ab may be two of multiple autoantibodies that contribute to the development of neuropathic pain. Future studies will evaluate additional autoantibody candidates and look at the possibility that adding to this panel of antibodies improves the predictive value.

E. Study impact in spinal cord injury

Results indicating the presence of GFAPab, and GFAP and/or CRMP2ab at 16 days are associated with the development of neuropathic pain within 6 months after SCI builds on experimental studies. Experimental mouse studies demonstrated that SCI causes autoantibody production and that autoantibodies impair recovery of function and worsen tissue damage.[5, 9] In SCI, however they did not demonstrate an association with the development of autoantibodies and the subsequent development of neuropathic pain. The discovery of autoantibodies reactive to GFAP and CRMP2 in SCI patients may lead to the development of new biomarkers for prediction of the development of neuropathic pain, and importantly, if determined as causal, new treatments for SCI-related neuropathic pain.

Pain has a serious impact on the quality of life for SCI patients experiencing neuropathic pain. Currently, there are no diagnostic tools available to predict who will develop chronic neuropathic pain, nor are there fully effective therapies available to treat this debilitating condition. Ultimately, the goal is that discovery of autoantibodies predictive of pain will not only detect those patients in need of intervention, but may also identify new areas of investigation that can create therapies to eliminate the onset of neuropathic pain and its associated morbidity.

APPENDIX

A. Protein A affinity columns

IgG purification was performed and affinity columns were created using recombinant protein A to covalently crosslink the antibody in the plasma sample. BCA and optical density measurements of washes and western blots and coomassie stained gels provided confirmation of successful IgG purification and antibody crosslinking to Protein A. The homogenized CNS tissue was applied the affinity column and the antigen/proteins were eluted with glycine. The antigen was then run on a Coomassie gel and Western blot. The eluted antigen was to be cut out of the Coomassie gel for identification by LC-MS/MS. Appendix Figure 1 shows GFAP eluted from the protein A affinity column made with custom anti-GFAP. Columns made with whole serum were not successful. Therefore, the 2-D gel approach was taken.

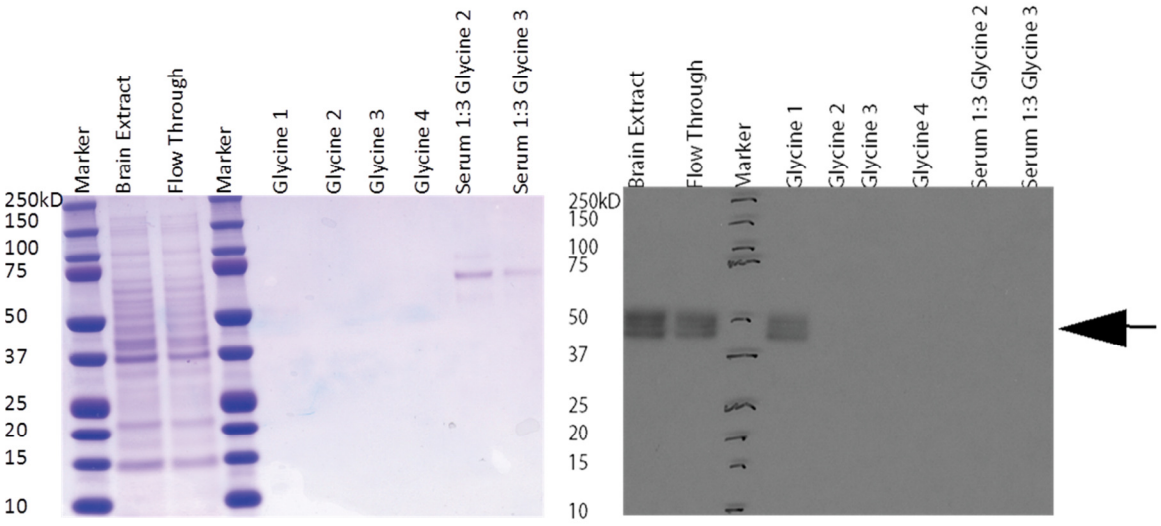
Methods

Antibody purification Antibody purification was performed with a Melon Gel IgG Spin Purification kit (Thermo Scientific, Rockford, IL). The kit binds high abundance proteins like albumin and transferrin using a mild buffer at physiological pH. It is amine-free. Columns were prepared with gel slurry (200µl) centrifuged and washed twice with 120 µl of purification buffer. Samples diluted with gel purification buffer (50µl sample: 450µl buffer) were added to the column and mixed for 5 minutes. Purified antibody was tested in a western blot, on a coomassie blue stain gel and after confirmation of protein it applied to the protein A affinity column.

Protein A affinity column Purified IgG was applied to protein A affinity columns(Pierce Protein A IgG Plus Orientation, Thermo Scientific, Rockford, IL) to crosslink

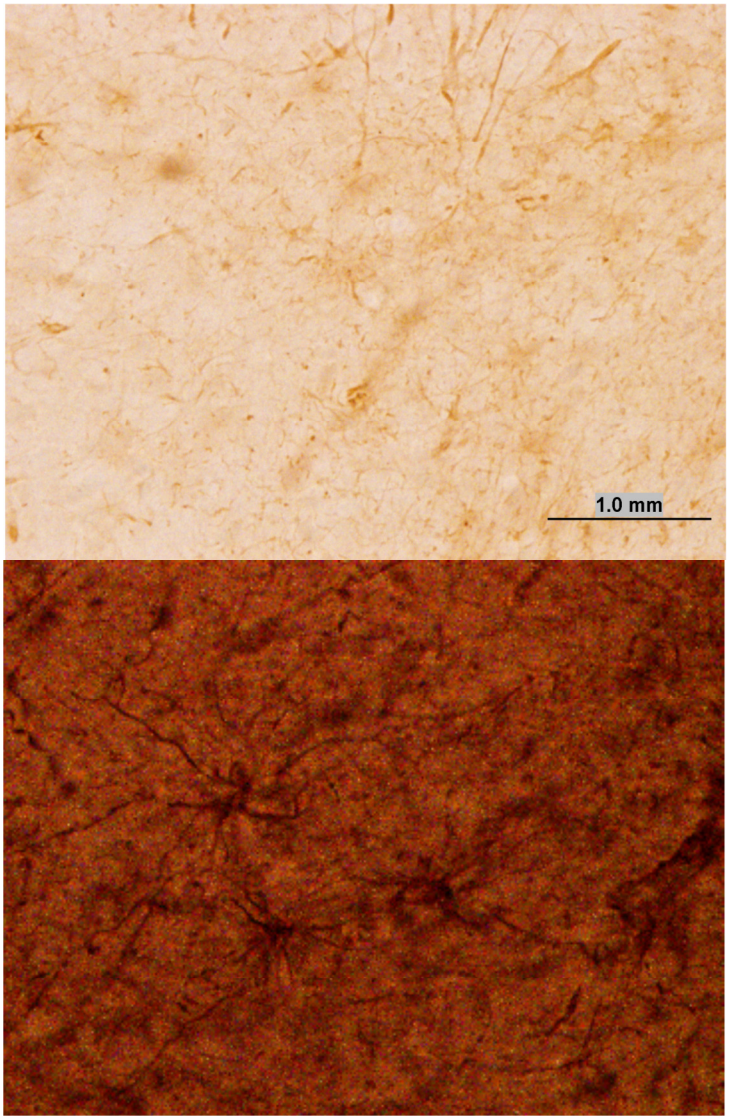
the antibody to protein A agarose. Purified IgG was diluted with antibody binding/wash buffer (1:1) and loaded on to equilibrated columns. The resin was re-suspended and mixed at room temperature for one hour. The resin was allowed to settle and solution pass through was collected for analysis of binding efficiency. Because binding efficiency was insufficient as measured by BCA and spectrophotometry, the process was repeated, but instead the resin and sample were incubated overnight at -4℃. Binding efficiency was confirmed with spectrophotometry. The columns were washed and Crosslinking buffer was prepared with DSS dissolved in DMSO and immediately applied to the antibody-bound column. The column was incubated for one hour for one hour on a rotator. The resin was washed with crosslinking buffer and then blocked with blocking buffer to block any remaining non-reacted NHS-ester groups. Resin was re-suspended and mixed. IgG elution buffer was applied to elute any IgG that was not covalently bound to protein A. The column was washed and completed and stored in a 0.02% sodium azide PBS solution at 4℃ for future use. Samples coming off the column at each step were saved and efficiency was evaluated by spectrophotometry.

Results



Coomassie gel (left) and western blot (right) of Glial Fibrillary Acidic Protein (GFAP) antibody affinity column (GFAP, MW 50kD, arrow). Affinity columns (AminoLink) for GFAP were created. Starting brain extract (50ul plus 450ul PBST) was incubated on the column. Flow through shows the brain proteins that were not retained on the column. Glycine 1 was the first elution from the column showing the antigen (GFAP) that was bound to the anti-GFAP column. Western blot was probed with a custom anti-GFAP primary antibody (1:6000) and alkaline phosphatase secondary anti-rabbit antibody. As anticipated, GFAP antigen is visible on the western blot that was probed with custom anti-GFAP (arrow). Serum elution from healthy volunteer serum was not expected to and did not show GFAP. When the columns were made using purified IgG from patient plasma as the antibody bound to the protein A agar, the proteins came off in the flow through, but did not come off on the glycine elution suggesting binding was not strong enough (not shown).

B. Immunohistochemistry (IHC) performed on monkey brain tissue.



Immunohistochemistry on monkey brain tissue. Monkey brain tissue stained with human plasma taken 16±7 days post-SCI (top). Faint staining of astrocytes can be seen. The same tissue probed with a custom anti-GFAP antibody showing reactive astrocytes (bottom). Similar to Ishida et al., the autoantibody was not clearly detectable using IHC. As suggested by them, this may be that the epitope of the antigen is concealed in situ because of intermolecular or intramolecular conformation. [195]

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Vita

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